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A Study on Microbial Ecology in Permanently Ice-covered Lakes of McMurdo Dry Valleys, Antarctica

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권미예

A Study on Microbial Ecology in Permanently Ice-covered Lakes of McMurdo Dry Valleys, Antarctica

Miye Kwon

Advisor: Professor Sang Jong Kim, Ph.D.

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Seoul National University

ABSTRACT

Antarctica has been considered as pristine and harsh environment in respect to microbes because of the extreme conditions. Perennially ice-covered lakes (Lake Fryxell, Lake Miers, and Lake Bonney) in the McMurdo Dry Valleys, Antarctica, are chemically stratified with depth and have distinct biological gradients. Despite of long-term research on these unique environments, the information on the structure of the microbial communities in the water columns of these lakes are scarce.

In this study, bacterial diversity in the ice-covered lakes in Antarctica was examined by 16S rRNA gene-based pyrosequencing. Distinct communities were present in each lake, reflecting the unique biogeochemical characteristics of these environments. Further, certain bacterial lineages were exclusively confined to specific depths within each lake. For example, candidate division WM88 occurred solely at a depth of 15 m in Lake Fryxell, whereas unknown lineages of *Chlorobi* were found only at a depth of 18 m in Lake Miers, and two distinct classes of *Firmicutes* inhabited East and West Lobe Bonney at depth of 30 m. Redundancy analysis revealed that community variation of bacterioplankton could be explained by the distinct conditions of each lake and depth; in particular, assemblages from layers beneath the chemocline had biogeochemical associations that differed from those in the upper layers.

Despite of the fact that sulfate reduction has been actively detected,

little is known about diversity of sulfate reducing bacteria in Lake Fryxell. Less than 1% of total bacteria are known to be culturable. By application of culture-independent approach such as metagenomics, not only full extent of bacterial diversity but also their ecological function could be obtained. Three draft genomes were recovered from metagenome data as sulfate reduction-related bacteria, two belonging to *Deltaproteobacteria* and one belonging to candidate division WM88. *Deltaproteobacteria* was a key player for sulfate reduction in Lake Fryxell whereas candidate division WM88 was indirectly related to the reaction of sulfate reduction. Other metabolic pathways were also investigated, and it was revealed that they were versatile in acquisition and usage of various carbon sources and energy conservation.

Another aspect of sulfate reducing bacteria in the bottom of Lake Fryxell was observed based on microcosm study as a culture-dependent approach. Through performing microcosm study using the lake water as a medium, previously uncultivated sulfate reducing bacteria were expected to be recovered from Lake Fryxell. From the microcosm culture, the genus *Desulfosporosinus* belonging to the phylum *Firmicutes* was surprisingly enriched in large proportion under specific conditions with low concentration of carbon source and sulfate. This genus has gained attention for the ability of sulfate reduction with high efficiency albeit low abundance in natural environments, which is known as ‘rare biosphere’.

The findings of patterns of bacterial community composition and their

ecological functions, including sulfate reduction, may represent adaptation of bacteria to the extreme and unique biogeochemical gradients of ice-covered lakes in the McMurdo Dry Valleys. Also, novel results may provide further insights into the ecology and evolution of bacteria inhabiting the ice-covered lakes of Antarctica.

Keywords: bacterial diversity, niche-specialization, sulfate-reducing bacteria, pyrosequecning, metagenome, microcosm, Fryxell, Ice-covered lakes, Antarctica,

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CONTENTS

ABBREVIATIONS

Dsr: Dissimilatory sulfite reductase

ELB: East Lobe Bonney

FRX: Fryxell

HOR: Hoare

MCM: McMurdo

MCM-LTER: McMurdo Long Term Ecological Research

MGC: Metagenome cluter

MIE: Miers

NCBI: National center of biotechnology information

OTU: Operational taxonomic unit

rRNA: Ribosomal ribonucleic acid

SRB: Sulfate reducing bacteria

WLB: West Lobe Bonney

3MA: > 3.0 µm bacteria

3MB: 3.0-0.2 µm bacteria

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CHAPTER 1.

General introduction:

Bacterial diversity and geochemistry in the ice-covered lakes, Antarctica

1.1. General information of McMurdo Dry Valleys, Antarctica

1.1.1. Antarctica and McMurdo Dry Valleys

Antarctica is the fifth-largest continent with about 14×10^6 km^2 in area, and is divided into two provinces, West and East Antarctica, by the Transantarctic Mountains (Cavicchioli, 2015) (Figure 1.1). The continent contains about 70% of the world's freshwater, which is covered by ice sheet of 1.6 km in thickness. The continent has the windiest, coldest, and driest environmental condition on the earth. Moreover, for past decades, many scientists have considered the climate change in Antarctica to be highly related to increase of air temperature on the earth. It has been impacting the temperatures in the Antarctic Peninsula during the 20^{th} century (Doran *et al.*, 2002). In a preceding research, microorganisms were shown to be functional as a bioindicators of climate change under the extreme environmental conditions (Pointing *et al.*, 2009). Therefore, the research of microbes and their ecology in Antarctica is imperative to understand biosphere of the earth.

Above all, Antarctica has been considered as an encyclopedia of the earth providing valuable records of past climates. For example, bubbles in the ice core (Legrand *et al.*, 1988, Petit *et al.*, 1999) and soils in the sediment core (Blunier *et al.*, 1998) recorded not only the chemistry of ancient air but also environmental changes that took place in the history. In addition, rocks

(Wierzchos & Ascaso, 2002) in the Antarctic continent have carried fossils as a key record to the global climate system as well. Antarctica is the “the best natural laboratory of natural selection” to the researcher.

McMurdo Dry Valleys (MDVs) of Southern Victoria Land constitute of area of ~4,800 km^2 encompassing the largest ice-free region about 1% of the Antarctic continent containing four major valleys, two of Wright and Victoria in north, Taylor in south, Miers in further south (Cavicchioli, 2015). The desert of MDVs has been persisted for 15 million years. The ice free region of MDVs is caused by complex reasons such as low annual precipitation (~3 cm), low annual mean temperatures approximately -14°C to -30°C, katabatic winds descended from the polar plateau, solar heating and extended periods of darkness, sunlight, and etc. (Clow *et al.*, 1988). Interestingly, scientists of National Aeronautics and Space Administration (NASA) conducted landing tests of the Viking spacecraft on the MDVs before sending the Viking spacecraft to Mars in 1970s because two environments share similar extreme conditions. The MDVs are consisted of glaciers, organic matter-deficient soils, ephemeral streams, eroded rocks by wind, and a mosaic of permanently ice-covered lakes. The ice-covered lakes have been formed as a result of trapped coastal water by isostatic rebound (Hodgson *et al.*, 2001), then these closed coastal lakes have been evolved to be saline because of the evaporative concentration of salts over time (Pickard *et al.*, 1986, Spigel & Priscu, 1998).

1.1.2. Ice-covered lakes in McMurdo Dry Valleys

The presence of ice cover is a unique feature of MDVs lakes, varying from 2 to 6 m of ice thickness. The permanent ice cover plays an important role to biogeochemistry of the water column such as inhibition of wind induced turbulence, chemical stratification, stable water column (Green & Lyons, 2009), low light penetration (Priscu, 1991), and restricted exchange between surface water and the atmosphere (Prisu *et al.*, 1996). Also, cryoconite of the ice cover provide a habitat for microorganisms (Murray *et al.*, 2012). During the austral summer, moats of the lakes melt along the shorelines, and the ice cover regularly shows a crack (Tyler *et al.*, 1998). Nutrient input occurs by the stream flow of melt water from glaciers. Therefore, to observe microbial ecology during the seasonal changes of the Antarctic freshwater lakes, especially, the ice-covered lakes of Taylor Valley (TV) and Miers Valleys (MV) have been focused for more than 20 years of research by McMurdo Long Term Ecological Research (MCM LTER) project (www.mcmlter.org).

The four ice-covered lakes of TV are Lake Fryxell (FRX), Lake Hoare (HOR), and Lake East Lobe Bonney (ELB) and West Lobe Bonney (WLB), and the MV includes one lake; Lake Miers (MIE) (Figure 1.2 and Figure 1.3). These lakes can be categorized into three groups by salinity; FRX as brackish, HOR and MIE as freshwater, and ELB and WLB as hypersaline. These ice covered lakes are closed-basin system, and the only way of hydrologic loss is by ablation. Despite the hydrological and morphological similarity, the lakes

have been presented different biogeochemical features. Wilson (1981) proposed that physico-chemical characteristics of the saline lakes could be affected by landscape position and their evaporitic histories (Wilson, 1981).

Lake Fryxell (FRX) is located at the northeastern end of the TV at 77.37°S, 163.09°E surrounded by Canada and Commonwealth Glacier and directly connected with thirteen streams. FRX has a surface area of 7.06 km^2 and a volume of water of 25.2 × 10^6 m^3 (Spigel & Priscu, 1998) covered with about 4.3 m thick permanent ice cover. FRX is the shallowest lake with about 18 m depth with a chemocline at 10 m. The upper water is supersaturated with oxygen, but the water column exhibits brackish and anoxic conditions under the chemocline. The lake has a stable water column, and showed stratification by temperature. Interestingly, concentration of sulfate decreased while that of hydrogen sulfide (H_2S) increased with depth, exceeding 1 mM near the sediment (Karr *et al.*, 2005, Saxton *et al.*, 2016). Methanogenesis also occurs producing methane in sediments (Smith *et al.*, 1993)

Lake Hoare (HOR) is fed by the Canada Glacier, and located at 77°38'S, 162°51'E at the west of FRX. The Canada Glacier blocked the connection between FRX and HOR. HOR has a surface area of 17.5 km^2 and a volume of water with 1.9 × 10^6 m^3. There is no stratification in HOR, and it is about 30 m depth with about 3.8 m of the ice thickness. The surface of the ice cover is very rugged and contains a bulk of sand blew by the wind from the shore. HOR is the most oligotrophic lake with low solute levels, low dissolved nitrate, high

pH, and high oxygen detecting even at 24 m depth (Spigel and Priscu, 1998). The concentration of sodium increased with depth ranging from 54 mg l^{-1} to 170 mg l^{-1} (McKnight *et al.*, 1993).

Lake Bonney (LB) is located at the southwest end of the TV at 77.43°S, 162.20°E, which is dividing two lobes into west (West Lobe of Bonney;WLB) and east (East Lobe of Bonney;ELB). WLB has melt water sources from the two glaciers; Rhone and Taylor Glaciers, while ELB is fed by meltwaters from three of Glaciers; Hughes, Sollas, and Matterhorn, and also from WLB by the sill. Two lobes are connected by a narrow channel about 13 m wide, and the water column of two lobes present chemical stratification due to the 4 m of permanent ice cover. Both lakes are about 40 m deep with a chemocline at 15 m depth. However, they are considered as different lakes due to very different bio- and phygeochemistric characteristics (Green & Lyons, 2009) by long separation and evaporation history (Glatz *et al.*, 2006). For example, WLB exhibits the concentration of very low nitrate and high ammonium in anoxic water layer, and it suggests that aerobic nitrifying bacteria and anaerobic denitrifying bacteria, respectively, play a role around the oxic/anoxic transition (Ward & Priscu, 1997). Unlikely, ELB shows no denitrification activity under the chemocline showing very high concentration of nitrate, nitrite and nitrous oxide which are shown on the fossil record by microorganism (Prisu *et al.*, 1996). Also, the bottom water shows higher concentration of salinity than seawater. ELB is more saline than WLB because ELB has active evaporation,

while WLB is directly affected by Taylor Glacier. Interestingly, Blood Falls are discharged from a tongue of the Taylor Glacier, which is in direct contact with WLB and is a potential source of salt (Black *et al.*, 1965) to WLB. Blood Falls are hypersaline water containing high iron and sulfate concentrations (Mikucki & Priscu, 2007). These ionic compositions, which resembled seawater, of Blood Falls are possibility the result of interactions between iron-rich bedrock and subglacial brine originated from marine incursion to MDVs (Lyons *et al.*, 2005).

The ice-covered Lake Miers (MIE) is located in a depression in the moraine-covered Miers Valley with 1.5 km long and 0.7 km in width (Bell, 1967). The inflow of MIE is meltwater inserted through two streams from Miers and Adams Glaciers during summer, and the outflows through the Miers River into McMurdo Sound. MIE is covered with the ice that is about 3.8 m thick and contains rock debris which could be trapped by new ice after blown from the lake shores (Bell, 1967). MIE is categorized as a freshwater, and only NH_4^+ present as a nitrogen component. Also, the lowest depth is shown to have high concentration of Ca^{2+} eluted from calcite layer of lake bed. Interestingly, the temperature of the water column rose from 4°C at 5 m to 5°C at 20 m (Bell, 1967).

Among five lakes, LB and FRX have chemoclines sectioning oxygen-rich, surface freshwaters from saline waters in the bottom of the lakes. The deep water of FRX are strongly anaerobic and is the only lake that has high levels of

sulfide. LB is strongly phosphorous deficient whereas the other lakes have shown to be both N and P deficient (Priscu, 1995, Dore & Priscu, 2001). For nitrogen chemistry in FRX and WLB, NO_3^- is depleted in the surface, but NH_4^+ is high in anoxic deeper layer. Detailed profiles of inorganic nutrients and dissolved organic carbon can be found in previous researches (Priscu, 1995, Priscu, 1997).

1.2. Microbial diversity and their pattern in the ice-covered lakes

1.2.1. Bacterial diversity and community structure in the ice-covered lakes

Microbes are cosmopolitan organism in a wide range of habitats on the earth from the terrestrial and aquatic ecosystem to even extreme environments, and they are diversified into over 4-6 × 10^{30} of microbial cells (Nisbet & Sleep, 2001). The McMurdo (MCM) lakes are known as a "complex consortium of interacting organisms" by microbial processes and planktonic food webs in spite of harsh conditions with low nutrient and low light levels (Laybourn-Parry, 1997, Priscu *et al.*, 1999). A variety of bacterial communities have been reported in the ice-covered lakes such as *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Cyanobacteria*, and many candidate phyla using several methodologies of culture dependent/independent such as functional

gene screening, cloning, DGGE, and high-throughput sequencing. Dominant bacteria are shared among the ice-covered lakes (Kwon *et al.*, 2017), and most of identified bacteria belong to the psychrophilic or cold-adapted bacteria (Sattley & Madigan, 2006, Bielewicz *et al.*, 2011, Tang *et al.*, 2013, Vick-Majors *et al.*, 2013, Dolhi *et al.*, 2015, Bowman *et al.*, 2016, Saxton *et al.*, 2016).

Furthermore, certain bacteria were only found in particular lakes. In FRX, the phototrophic purple bacteria were observed and have been cultivated with gas vesicles, which would help to be positioned at specific depths in water column (Karr *et al.*, 2003). Methanogenic, chemolithotropic sulfur-oxidizing, and sulfate reducing bacteria were examined from the anoxic zone of FRX (Karr *et al.*, 2006, Sattley & Madigan, 2006). Several cold-active chemoorganotrophic bacteria (the genera *Polaromonas*, *Psychrobacter, Marinobacter*,*Caulobacter*, and etc.) have been found in HOR and LB (Clocksin *et al.*, 2007, Mikucki & Priscu, 2007). Owing to the different geochemistry conditions, aerobic nitrifying bacteria and anaerobic denitrifying bacteria exhibited the boundary of the oxic/anoxic zone in WLB (Ward & Priscu, 1997), respectively. Also, pigmented colonies were recovered from the enrichment of water column in HOR, which protected the cells from damages caused by oxygen supersaturated conditions (Clocksin *et al.*, 2007).

Also, microbial community has been composed of various microorganisms such as protozoa, fungi, algae, and cyanobacteria, and their composition ratio has been varied depending on the incident of quantity and

quality of Photosynthetically Active Radiation (PAR). (Wharton Jr *et al.*, 1983). In HOR, diatoms and cyanobacteria (*Phormidium* sp. and *Lyngbya* sp.) dominate, which generate oxygen by photosynthesis (Spaulding *et al.*, 1997, Vopel & Hawes, 2006). Testate rhizopods are also found, and they could be paleo-indicators owing to their preservation ability (Scott & Asioli, 2014).

However, the most abundantly observed species in the environment constitute only minor portion in actual microbial world because there still are un-cultivated microbes that exist in the environment (Galand *et al.*, 2009). In recent years, high number of novel microbes were identified and introduced through molecular methods such as sequencing technology, outnumbering microbes that were physically isolated, thereby supporting the fact that diversity of microbes is much higher than they had been observed until now (Rappé & Giovannoni, 2003, Pedrós-Alió, 2007). The hidden microbes in nature are usually present at low abundance (<1%) thereby they are called as 'rare biosphere' (Sogin *et al.*, 2006). These rare species could be a member of uncultivated species of 'candidate phylum' (McLean *et al.*, 2013, Nobu *et al.*, 2016), which are still estimated to be over 100 candidate phyla within the domain *Bacteria* (Baker & Dick, 2013, Yarza *et al.*, 2014). The research of the microbial ecology in extreme environments like Antarctica provides a prospect to discover novel microorganisms (Markert *et al.*, 2007). Furthermore, in previous studies, rare taxa were identified to play important ecological and functional roles in the environments such as anaerobic methane oxidation and

mineralization of organic compounds (Pester *et al.*, 2010, Pedrós-Alió, 2012).

1.2.2. Diversity of sulfate-reducing bacteria in the ice-covered lake Fryxell

Sulfate-reducing bacteria (SRB) play significant ecological role in the carbon and sulfur cycles within aquatic environment, mineralizing more than half of organic matter by dissimilatory sulfate reduction (Jørgensen, 1977). SRB can be divided into seven phylogenetic lineages, five in the domain Bacteria and two in the domain Archaea (Muyzer & Stams, 2008). In preceding studies, several generalizations have been summarized for the mechanism of sulfate transport by SRB: SRB showed a high-affinity to sulfate even in limited sulfate concentrations and the proton-motive force appeared to be important for nutrient uptake because the affinity system of proton/sulfate is high. Protons and Na^+ ions are used for symport of sulfate in freshwater and marine conditions, respectively (Ingvorsen & Jørgensen, 1984). SRB are usually found in freshwater environment with low concentrations of sulfate (between 10 μM and 750 μM) (Smith and Klug, 1981) whereas they are found in higher concentrations of sulfate (28 mM) in marine environments. Also, SRB are known as a rich source of cytochromes associating a wide range of hemes from one to sixteen (Moura *et al*. 1991).

Dissimilatory sulfate reduction is a form of anaerobic respiration, and intracellularly occurs in three steps: 1) sulfate (SO_4^{2-}), which is already

transported across the cytoplasmic membrane, is converted to adenosine 5'-phosphosulfate (APS) by ATP sulfurylase (Sat), consuming a single ATP molecule (Hansen, 1994). This step is a biochemical necessity because the redox couple of sulfate-sulfite is unfavorable, because it is too negative for the reduction by NADH or ferrodoxin to take place as the primary electron mediators (Muyzer & Stams, 2008). 2) APS is reduced to sulfite (SO_3^{2-}) by adenylyl-sulfate reductase (APS reductase; Apr), which contains FAD with Fe-S groups, requiring the input of 2 electrons (Muyzer & Stams, 2008). 3) In the final step, sulfite is reduced to sulfide (HS^-) by dissimilatory sulfite reductase (Dsr), requiring the input of 6 electrons (Grein *et al.*, 2013). The most important enzymes for sulfate reduction are two terminal reductases (AprAB and DsrAB). Also, two membrane complexes (QmoABC and DsrMKJOP) are essential for sulfate reduction pathway, which transport electrons and provide them to two terminal reductases (Pereira *et al.*, 2008).

Dissimilatory sulfite reductase (Dsr) of SRB is an important enzyme in final step of sulfate reduction, and possesses the $\alpha_2\beta_2$ tetramer with reduction active site at a siroheme linked to a 4Fe-4S cluster (Dahl *et al.*, 1993). The subunits of α and β are encoded by paralogous genes *dsrA* and *dsrB* in a single copy operon, respectively (Dahl *et al.*, 1993, Wagner *et al.*, 1998). The subunit of γ_2 encoded by DsrC gene is found only in a few organisms, which are related to the electron transfer process between an external electron donor and sulfite (Mander *et al.*, 2005). DsrAB enzymes are considered as very ancient, and are

best studied from SRB because they is conserved in all identified SRB (Pester *et al.*, 2012, Müller *et al.*, 2015). For these reasons, not only 16S rRNA but also DsrAB genes have been considered as congruent phylogenetic marker genes for SRB in environment samples (Loy *et al.*, 2002, Leloup *et al.*, 2006, Moreau *et al.*, 2010).

Virtually, however, many findings for SRB have been derived from a few strains (e.g. the genus *Desulfovibrio*). Notwithstanding the recent report for many species- or strain-specific differences, there is still poorly unclassified diversity of SRB and DsrAB genes in the Antarctic environments. By application of the DsrAB gene approach, it is possible to uncover extensive hidden diversity of SRB and DsrAB sequences from the extreme environments (Wagner *et al.*, 2005, Müller *et al.*, 2015).

1.3. Molecular technologies for microbial ecology

1.3.1. Molecular biological tools

Nucleic acid-based molecular approach has been widely applied for classification and identification of microbes (Pereira *et al.*, 2010). Ribosomal RNA gene, in particular, 16S rRNA gene for bacteria, has been used as biomarkers to reconstruct phylogenetic studies (Weisburg *et al.*, 1991) with more than a few advantages: 1) the hypervariable regions are highly conserved but distinguishable to classified bacterial taxa (Coenye & Vandamme, 2003), 2) the regions of the gene showed slow rates of evolution (Woese & Fox, 1977),

3) all bacteria contain 16S rRNA gene, and 4) the databases are well established to use and those include EzTaxon-e, Ribosomal Database Project (RDP), SILVA, and GreenGenes.

There are a variety of molecular tools to assess bacterial diversity based on 16S rRNA gene using fingerprinting (denaturing gradient gel electrophoresis; DGGE, amplified ribosomal intergenic space analysis; ARISA, terminal restriction fragment length polymorphism; T-RFLP and polymerase chain reaction; PCR), probe (Fluorescent in situ hybridization; FISH, Catalyzed reporter deposition; CARD-FISH) and molecular cloning of the partial/full length of 16S rRNA gene. Also, quantitative real-time PCR, so called real-time PCR or qPCR, can quantify target biomarkers using fluorescent dye (Fierer *et al.*, 2005) while sanger sequencing has imparted the information of presence or absence. However, although there are many benefits in the usage of 16S rRNA as a marker, there still is a drawback because the information based on 16S rRNA gene does not explain the actual activity of the bacterial cells. Also, fingerprinting and cloning techniques produce the relatively low resolution data, and do not cover all bacterial taxa. In addition, all process of work are not only time-consuming but also costly.

1.3.2. High-throughput deep sequencing

New sequencing techniques have opened opportunities to discover not yet detected and defined microbial communities. Next-generation sequencing

(NGS) is much cheaper and faster method to generate hundreds and thousands of nucleotide sequences than traditional sequencing methods using various platforms. 454 pyrosequencing based on 16S rRNA gene amplicon (Roche GS-FLX Titanium) produces about 500 Mb per single run with 500 bp of average read length, and the property of read length from the 454 platform is confirmed to identify microbial taxa (Liu *et al.*, 2008). Also, 16S rRNA gene amplicon sequencing with Illumina Miseq platform can yield much more reads which are about five million reads of 2 × 250 bp, and the paired-end reads can also be used for taxonomic analysis (Bartram *et al.*, 2011). Moreover, metagenome shotgun sequencing with Illumina Miseq platform allow us to reach much deeper world of microbial community yielding all nucleic acid sequences from sample (Kunin *et al.*, 2008). Metagenomes have been used to retrieve nearly-complete genomes of dominant population within the sample, especially the uncultured microbes (Tyson *et al.*, 2004, Kirkegaard *et al.*, 2016, Nobu *et al.*, 2016). These powerful advantages of metagenomics give critical clues of potential metabolic function for unveiled microbes in various environments (Albertsen *et al.*, 2013, Dodsworth *et al.*, 2013).

1.4. Objectives of this study

Many theoretical and empirical researches have been conducted on physicochemical and hydrodynamic characteristics in ice-covered lakes of McMurdo Dry Valleys, Antarctica. These data have been monitored over 20

years by MCM-LTER program. However, patterns of microbial diversity and distribution are still pooly understood with little investigation, despite of the important roles of microbes in the Antarctic lakes. In particular, biodiversity of Lake Miers in Miers Valley has been focused on only mineral soils, but not within the lake (Babalola *et al.*, 2009). Given the paucity of information on microbes, it is worth studying for microbial diversity and their metabolic abilities to better understand the ecosystem of the Antarctic lakes. The aim of this study was to test a hypothesis that many unexplored branches of the bacterial tree of life are yet be found under the unique environmental conditions within the lakes of Antarctica (Figure 1.4). Also, microbial communities would be governed by varied or particular environmental conditions, and specific mechanisms could be explained in response to those conditions.

Here, I performed the research with several questions under the hypothesis:

First, how do bacterial communities differ by physical factors such as lake, month, depth, and size-fractions? Are these differences represented as idiosyncratic bacterial taxa exclusively found in any specific category? How do possible links between depth profile of bacterial communities and geochemical factors exist?

Second, what kinds of metabolism and sulfate reduction-related metabolic potential exist in Lake Fryxell? Who is the key player for sulfate reduction in the bottom of Lake Fryxell?

Third, how could uncultivated sulfate-reducing bacteria be enriched from Lake Fryxell? How do enriched sulfate reducing bacteria in Lake Fryxell be differ from other environments?

To address these questions, I investigated the vertical bacterial community structure of the ice-covered lakes in the McMurdo Dry Valleys by 16S rRNA gene-based pyrosequencing. Then, I focused on sulfate reducing bacteria in the bottom depth of Lake Fryxell, which inferred more valuable factors with abiotic/biotic features, to analyze their potential function and to figure out effective enrichment conditions for not yet recovered sulfate-reducing bacteria using culture-independent and -dependent approaches, respectively.

Any resultant of biological data could be one of the driving forces beyond exploration of the Antarctic freshwater lakes. Also, all findings would mark another milestone in the Antarctic science, by providing a baseline in truncated and extreme biospheres.

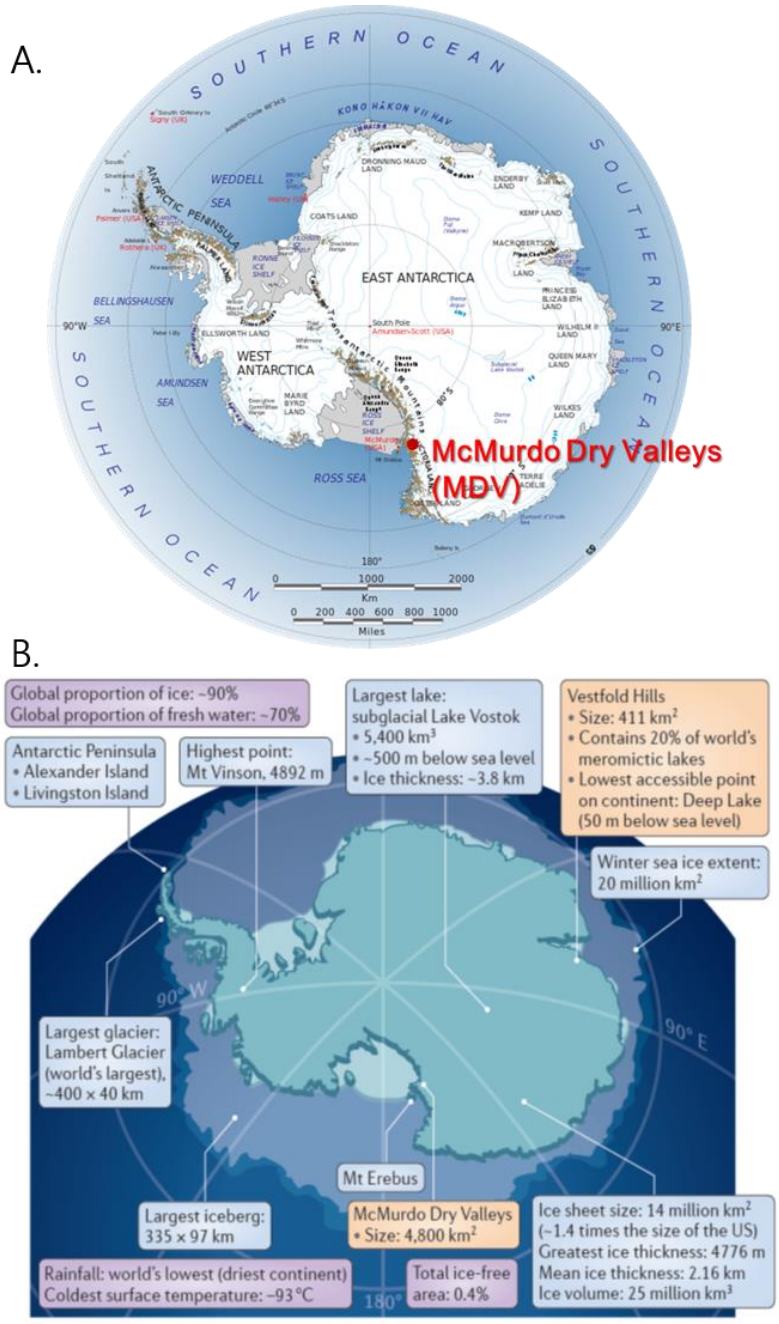

Figure 1.1. Overview of (A) the Antarctic continent and (B) features. Figure A and B were from website of Wikipedia and published paper **(Cavicchioli, 2015)**, respectively.

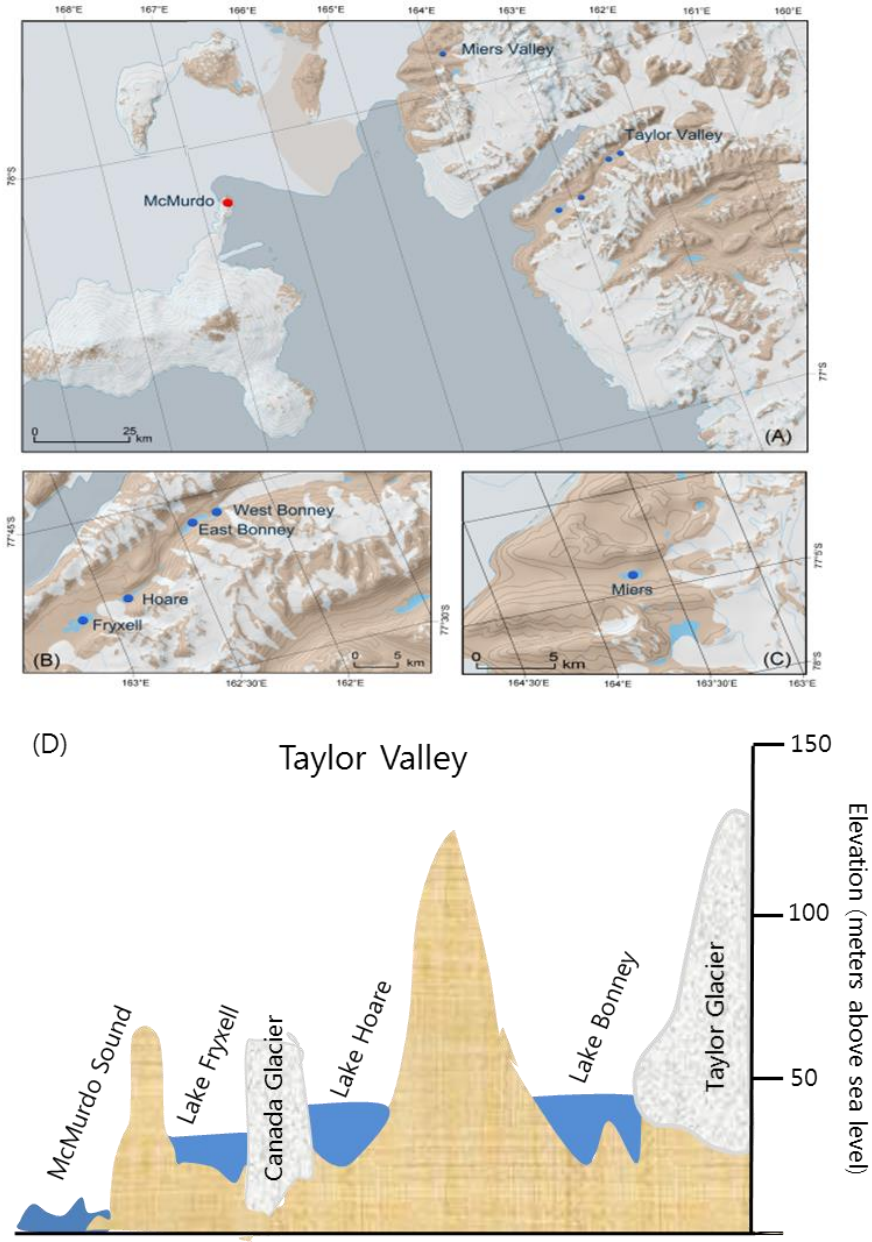

Figure 1.2. Map of (A) McMurdo Dry Valleys of Antarctica. (B) FRX, HOR, ELB and WLB are located in Taylor Valley, and (C) MIE in Miers Valley. (D) Vertical diagram of Taylor Valley.

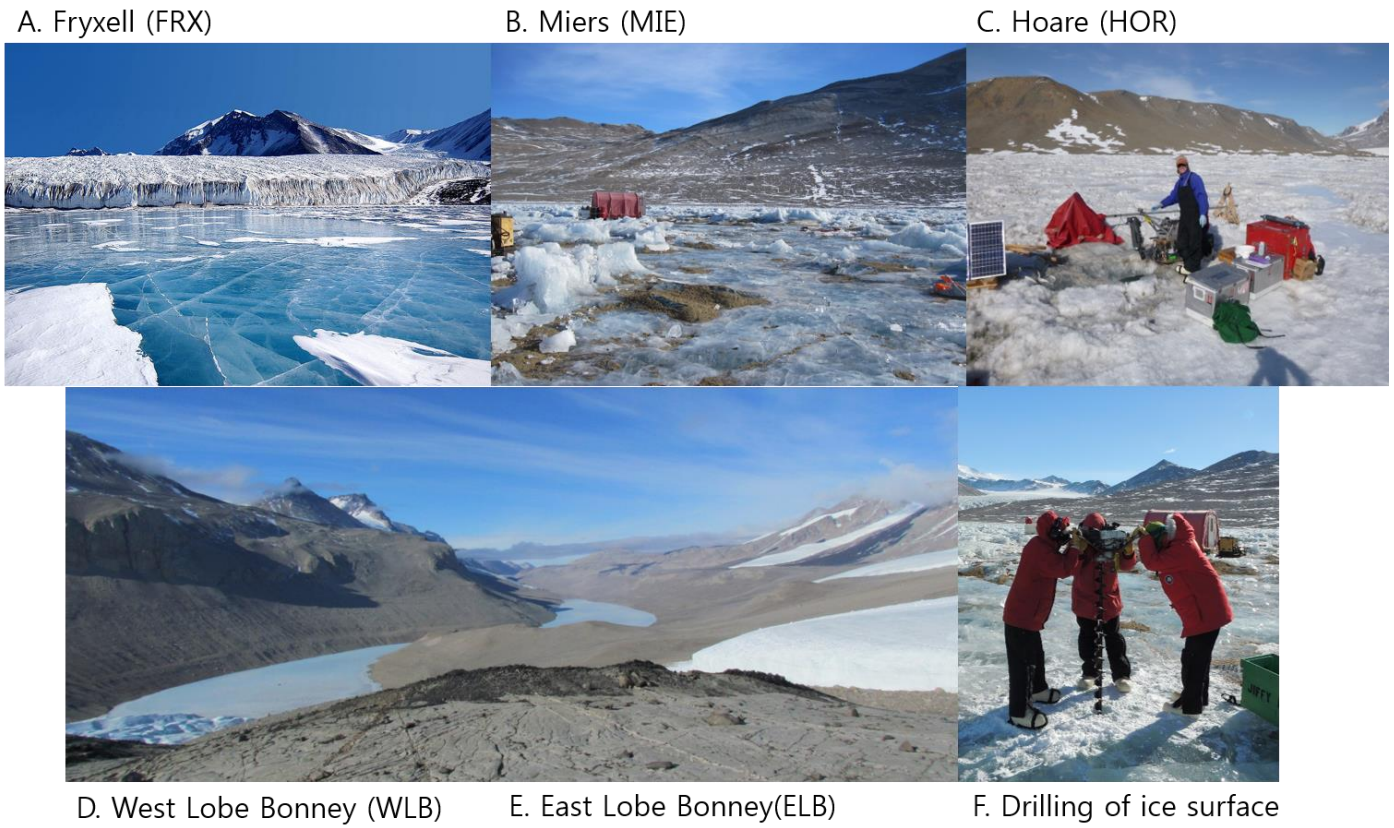

Figure 1.3. Landscape of ice-covered lakes in McMurdo Dry Valleys and drilling for sampling hole on the ice surface.

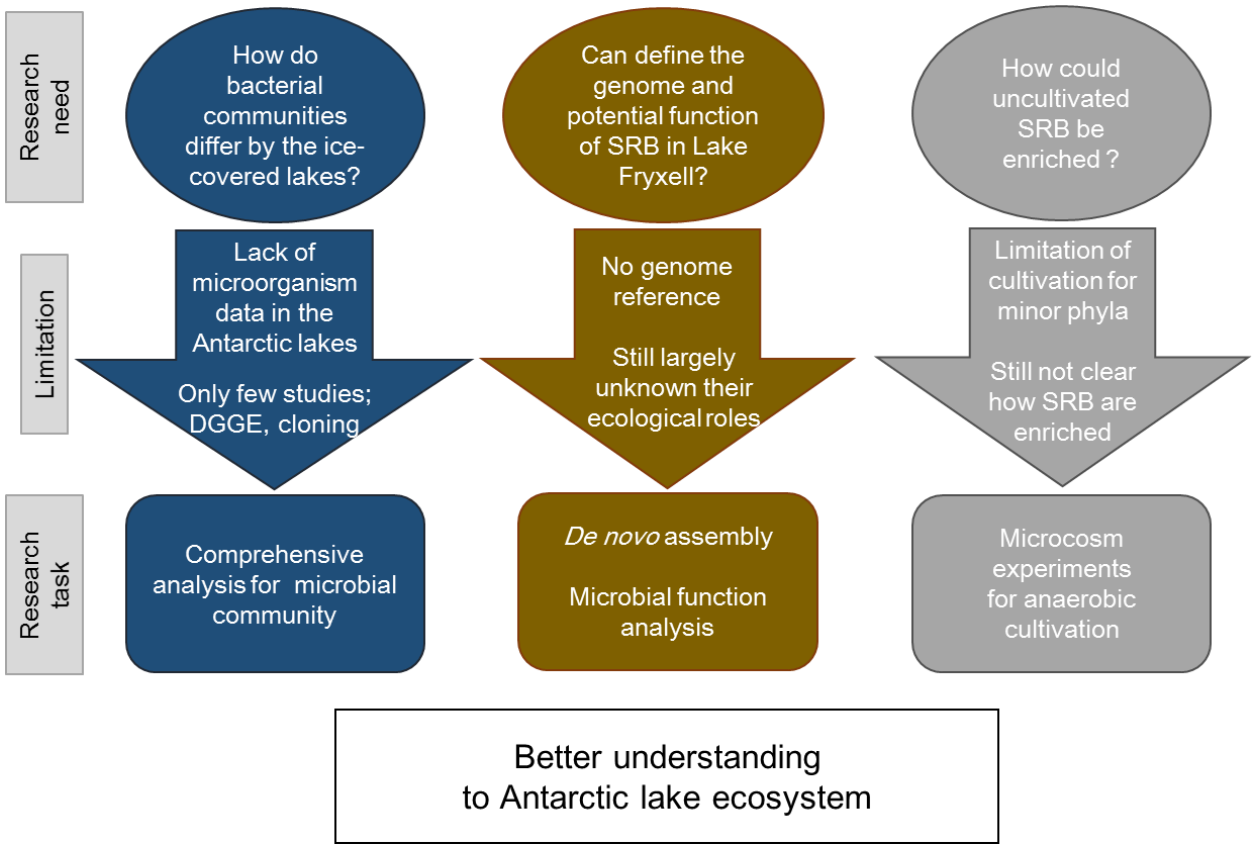

Figure 1.4. Research scheme with three questions

CHAPTER 2.

Bacterial community structure in permanently ice-covered lakes of McMurdo Dry Valleys, Antarctica

Contents of this article are modified from the published paper

"Niche specialization of bacteria in permanently ice-covered lakes of the McMurdo Dry Valleys, Antarctica"

Miye Kwon, Mincheol Kim, Cristina Takacs-Vesbach, Jaejin Lee, Soon Gyu Hong, Sang Jong Kim, John C Priscu, and Ok-Sun Kim

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2.1. Introduction

The McMurdo Dry Valleys (MDVs), located in Southern Victoria Land, Antarctica, is the coldest and driest desert on our planet. The MDVs are exposed to severe environmental conditions such as a mean annual temperature below 0°C, high levels of ultraviolet radiation, extremely low precipitation, extended periods of darkness, frequent freeze-thaw cycles and attenuated photosynthetically active radiation (PAR) (Lizottel & Priscu, 1994, Fountain *et al.*, 1999). Despite these conditions, numerous perennially ice-covered (3-6 m ice thickness) lakes occur across the valleys, most of which are chemically stratified and have no outflows. The permanent ice cover inhibits wind induced turbulence, which together with strong chemical stratification, results in extremely stable water columns with little vertical mixing (Priscu, 1995, Spigel & Priscu, 1998). Bowman *et al*. (2016) showed that these lakes are moderately productive and contain a diverse group of microplanktonic prokaryotes and eukaryotes (Bowman *et al.*, 2016).

The lakes of the MDVs have been isolated from each other and from exchange with atmosphere by their perennial ice covers for perhaps thousands of years (Lyons *et al.*, 1998, Poreda *et al.*, 2004). The strong chemoclines in many of the lakes effectively isolate the deep saline waters from the fresh surface waters (Spigel & Priscu, 1998). The deep saline waters of these lakes are often sub-oxic and contain unique geochemistries (Priscu, 1997, Lee *et al.*, 2004, Priscu *et al.*, 2008). These unique lake systems are dominated by bacteria,

comprising 30-60% of total microplankton biomass (Takacs & Priscu, 1998). Bacterial production is greatest just beneath the ice cover and a second peak in production occurs near the chemocline (Takacs & Priscu, 1998).

The microbial diversity of the MDV lakes has been surveyed by culture-dependent approaches (Karr *et al*., 2003; Sattley and Madigan, 2006) and more recently using culture-independent methods including DNA fingerprinting (e.g. DGGE, t-RFLP), cloning/sequencing targeting functional genes (Dolhi *et al*., 2015; Karr *et al*., 2005; Kong *et al*., 2012), taxonomic marker genes (e.g. rRNA genes) (Bielewicz *et al*., 2011; Mikucki and Priscu, 2007; Glatz *et al*., 2006; Tang *et al*., 2013) and 454 pyrosequencing (Vick-Majors *et al*., 2013; Bowman *et al*., 2016). The studies of Vick-Majors *et al*. (2013) and Glatz *et al*. (2006) revealed a portion of this diversity but concentrated mainly on one or two lakes and limited depths. Consequently, finer-scale taxa composition remains poorly understood.

The ice-covered lakes in Taylor Valley showed differ biogeochemical characteristics nonetheless they are linear location in the valley (Green and Lyons, 2009). Also, the lake in Miers valley showed similar biogeochemical characteristics with certain lakes in Taylor Valley as a freshwater (Bell, 1967). Owing to the logistical difficulties of an access, representative lakes were chosen for the study. The aim of this study is to compare the diversity and structure of bacterial communities between ice-covered MDV lakes to test the hypothesis that many uncharted branches in the bacterial tree of life will occur

under the unique environmental conditions within the lakes. Specific questions addressed in our study include: How do bacterial communities differ by lake, depth and size-fractions? Are any taxa limited to certain lakes or regions of the lakes? How do bacterial communities vary over the summer growth season, two months of continuous sunlight that are marked by changed biomass and productivity? Is the distribution of bacteria related to contemporary geochemistry within and between lakes?

To address these questions we investigated the vertical bacterial community structure of five ice-covered lakes from the MDVs using 16S rRNA gene-based pyrosequencing and related patterns of community composition and distribution to *in situ* conditions.

2.2. Materials and Methods

2.2.1. Site description and sample collection

The five lakes in our study lie in the Taylor (Fryxell, FRX; Hoare, HOR; East Lobe Bonney, ELB; and West Lobe Bonney, WLB) and Miers (Miers, MIE) Valleys and have been the focus of more than 20 years of research by the NSF funded McMurdo long term ecological research (MCM LTER) project (http://mcmlter.org) (Figure 2.1). FRX, HOR, ELB and WLB are hydraulically terminal lakes (i.e., they lack outflows) whereas MIE has a seasonal outflow to the sea during high water years. The lakes receive continuous sunlight during the summer and no sunlight between mid-April and late September (Bowman

et al., 2016; Morgan-Kiss *et al*., 2016).

A total of 40 samples were collected using Niskin bottle (4 l) with a workstation benchtop accessory. Lake samples were from depths representing the geochemically distinct water layers in each lake for pyrosequencing and the biogeochemical measurements in November and December in 2012, with the exception of HOR in November due to logistical constraints. Previous study has shown that most bacteria can be divided into particle-associated bacteria (>3.0 μm) and small (0.2 to 1.0 μm) and large (1.0 to 3.0 μm) free-living bacteria (Simon, 1985). To differentiate the particle-associated (>3.0 μm) and free-living bacterial assemblages (0.2 to 3.0 μm), we immediately size-fractionated collected water samples into > 3.0 μm and 3.0-0.2 μm size classes using sequential filtration through 3.0 μm (ADVANTEC, Japan) and 0.2 μm (Millipore, Germany) membrane filters at the site. We set >3.0 μm size filter as the upper limit of pore size because microbial cells in these lakes are known to be dominated by picoplankton (Kong *et al*., 2014). The filtered samples were transported from the lakes to McMurdo Station. The filters were stored at -20°C during shipment to South Korea where they were stored at -80°C until analysis.

2.2.2. Chemical analyses and primary production measurements

Samples were analyzed according to the procedures outlined by the MCM LTER method manual (http://www.mcmlter.org/data/mcm-lter-data-file-

format-protocols-database-submission) and briefly summarized below. Temperature and conductivity were measured with an SBE 25 Sealogger CTD. Dissolved oxygen (DO) was measured with the azide modification of the mini-Winkler titration, and underwater photosynthetically active radiation (UwPAR) was measured using a LI-COR LI-193SA spherical quantum sensor (LI-COR Biosciences) near local noon on the site. Macronutrients (NH_4^+, NO_3^-, NO_2^-) were analyzed as described by Priscu (1995). Dissolved organic carbon (DOC) was determined using a Shimadzu TOC-V series total organic carbon analyzer. All DOC samples were filtered through precombusted and acid-leached 25 mm GF/F filters and acidified to pH = 2 with 6N HCl to eliminate inorganic carbon. Phytoplankton primary production (PPR) was measured by incubating duplicate light and a single dark sample *in situ* for 24 h with ^{14}C-labeled bicarbonate. Heterotrophic bacterial productivity (BP) was measured using [^3H]methyl-thymidine incorporation into DNA.

2.2.3. Pyrosequencing and data analyses

Genomic DNA was extracted from half of each filter membrane (after ~1 L has passed through them) using FastDNA® SPIN kit (MP Biomedicals, Illkirch, France) following the manufacturer's instructions. Extracted DNA from 80 filter samples were amplified targeting the V1-V3 region of 16S rRNA gene using barcoded fusion primers (27F: 5'-X-AC-AGAGTTTGATCMTGGCTCAG-3' and 519R: 5'-X-AC-

GWATTACCGCGGCKGCTG-3' where 'X' denotes 8-bp error-corrected barcodes and 'AC' is 2-bp linker sequence) (Lane, 1991). Briefly, the PCR was performed in a total volume of 50 µl containing 2 µl of DNA, 0.5 µl of each primer (10 pmol), 1 µl of *Taq* polymerase (2.5U/ µl) (GeneAll, Korea), 4 µl of dNTP mix (2.5 mM each), and 5 µl of 10X buffer provided with the enzyme, under the following conditions: an initial denaturation step at 94°C for 5 min followed by 25 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1.5 min. PCR products could not be recovered from two 0.2 µm-filtered samples of 30 m (Nov) and 30 m (Dec) in ELB. The 454 adapter sequence was incorporated into the purified PCR product and pyrosequencing was performed by Macrogen (Seoul, Korea) using 454 GS FLX+ Sequencing System (Roche, CT, USA). Barcode, linker, and primer sequences were trimmed and low-quality sequences were removed from the dataset using PyroTrimmer (Oh *et al*., 2012). The quality-filtered sequences were further processed following the 454 SOP using mothur (Schloss *et al.*, 2009). The resultant partial 16S rRNA gene sequences were clustered into operational taxonomic units (OTUs) by applying 97% similarity cutoff. Singleton OTUs were excluded from subsequent analyses to reduce the likely artifacts of PCR or sequencing errors. Taxonomic classification was performed using the Naïve Bayesian Classifier implemented in mothur against the EzTaxon-e database (http://eztaxon-e.ezbiocloud.net) (Kim *et al*., 2012). All 16S rRNA gene sequences that originated from archaea,

chloroplast and mitochondria were removed. Raw reads were submitted to the NCBI SRA database with an accession number of PRJNA276598.

2.2.4. Quantitative PCR

To estimate the bacterial rRNA gene abundance, we performed quantitative PCR using a CFX96 qPCR System (Bio-Rad, Hercules, CA) with SYBR Green as the fluorescent reporter (Bio-Rad, USA) by quantifying bacterial 16S rRNA gene copies. Partial 16S rRNA genes were amplified using the bacteria-specific primer set, 341f (5'-CCTACGGGAGGCAGCAG-3') and 797r (5'-GGACTACCAGGGTCTAATCCTGTT-3') (Lane, 1991; Nadkarni *et al.*, 2002). The amplification followed a 3-step PCR: 44 cycles with denaturation at 94°C for 25 s, primer annealing at 64.5°C for 25 s, and extension at 72°C for 25 s. Two independent real-time PCR assays were performed on each sample. The standard curves were created using a tenfold dilution series of plasmids containing the bacterial 16S rRNA gene from environmental samples and R square for standard curves was 0.99. For each sample tenfold dilution series ranging from 10^{-5} to 10^{-9} were run in triplicate. Negative controls containing no DNA template were also run in triplicate. It should be noted that the quantification of 16S rRNA gene copy number does not provide accurate estimates for bacterial abundance because the rRNA gene copy number varies between bacterial taxa and primers used for qPCR do not cover entire bacterial population. However, qPCR assay has been frequently used in many microbial

ecology studies due to its reproducibility (Fierer *et al*., 2005; Takahashi *et al*., 2014).

2.2.5. 16S rRNA gene cloning, sequencing and phylogenetic analysis

To retrieve better phylogenetic information about unknown WM88 lineages found in FRX, DNA extracted from 3.0 μm-filtered FRX sample at 15 m was used for clone library construction targeting the full-length bacterial 16S rRNA gene sequence. PCR was performed using the primer set 27f and 1492r with the same reaction condition as described above (Lane, 1991). The PCR product was cloned into the vector using the TOPO TA cloning kit (Invitrogen, CA, USA) and fifty were randomly selected clones were sequenced using M13F/M13R primers with ABI 3700 capillary sequencer (Applied Biosystems, CA, USA). Putative chimeric sequences were deleted using chimera.uchime implemented in mothur based on the chimera-free SILVA gold database (v.123).

Representative 16S rRNA gene sequences for each of bacterial phyla were downloaded from EzTaxon-e database (42 phyla and 911 sequences) and were aligned against a high-quality reference alignment from EzTaxon-e using SINA v1.2.11 (Pruesse *et al*., 2012). The alignment quality was manually checked based on the secondary structure of 16S rRNA using jPhydit (Jeon *et al*., 2005). A phylogenetic tree was constructed using maximum likelihood (ML) analysis with the GTR + Γ model of nucleotide substitution in RAxML v8.2.9

(Stamatakis, 2014). The reliability of the tree topology was assessed by generating 100 bootstrap replicates using the -f a option, which enables a rapid bootstrap analysis and search for the best-scoring ML tree in RAxML. Bootstrap convergence was tested using the -I autoMRE option and convergence was achieved at 300 replicates. We then performed a new rapid bootstrap analysis with 1,000 replicates to obtain stable support values. 16S rRNA gene sequences obtained in this study were deposited in GenBank under accession numbers KP862888 to KP862894.

2.2.6. Statistical analyses

The resultant high-quality reads were standardized to 562 per sample by randomly subsampling reads 100 times before comparing the level of diversity among samples. Diversity indices were generated based on OTUs defined at 97% sequence similarity of 16S rRNA gene using mothur. Bray-Curtis dissimilarities were calculated using a hellinger-transformed OTU matrix and non-metric multidimensional scaling (NMDS) analysis was performed based on the dissimilarity matrix using PRIMER 6+ (Clarke and Gorley, 2006). In order to test if bacterial community compositions significantly differ by lakes and depths, a permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) was performed with 999 permutations using the 'adonis' function in the vegan R package (Oksanen *et al*., 2015).

Distance-based linear models (DISTLM) were used to determine the

relationship between environmental variables and bacterial community structure by partitioning variation in community structure according to a multiple regression model (Legendre and Anderson, 1999). Prior to DISTLM, the distribution of each environmental variable was plotted and normalizing transformations were performed on skewed variables according to the 'Draftsman plot' result in PRIMER 6+. Month (Nov and Dec) was transformed into dummy variable, depth, UwPAR, AmbPAR, Chl a and PPR were square-root transformed, and NH_4^+, NO_3^-, NO_2^-, DOC, DIC, BP, SO_4^{2-}, conductivity, Na, K, Mg, Ca and Cl are log-transformed, and the rest of variables were left untransformed. Highly correlated variables (Pearson's $|r| > 0.90$) in each lake were excluded by testing multicollinearity between predictor variables. Forward selection of transformed environmental variables was used to determine how much of the variation in community structure was explained by an individual variable alone or together with other variables and the best model was selected based on adjusted R^2 values of each model. The combination of environmental variables that best explained the community variation was selected and graphically represented as distance-based redundancy analysis (db-RDA). Basic statistical analyses and plotting were performed in R version 3.0.2 (R Core Team, 2013).

2.3. Results

2.3.1. Biogeochemical characteristics in five lakes of McMurdo dry valleys

Biogeophysical characteristics of the lakes determined during the austral summer are presented in Table 2.1 and 2.2. These data reveal the strong vertical gradients in ionic strength within each lake and the differences among lakes. Oxygen levels above the chemocline are all above air saturation whereas deeper waters are typically suboxic. Only the deep waters of FRX were highly anoxic and contain high levels of hydrogen sulfide (~1.8 mM). All lakes have deep chlorophyll-a layers and corresponding high levels of primary production located just above the chemoclines. Bacterial productivity often peaked with chlorophyll-a (Chl a) and phytoplankton productivity maxima. DOC and inorganic N and P were always higher in the deep saline waters, reaching mM levels in several of the lakes.

2.3.2. Distribution and relative abundance of bacterial biomass

Quantification of bacterial 16S rRNA gene abundance (which represents a measure of biomass assuming that all organisms have a uniform copy number of the gene) during Nov and Dec was estimated using qPCR in the two size fractions (3.0 and 0.2 μm) (Figure 2.2). Overall, vertical trends in bacterial

biomass differed by lake, size-fraction and month. In FRX, bacterial biomass increased with depth in all samples except for the 0.2 µm-Nov samples, which peaked at 9 m followed by a biomass decline to 15 m. In MIE, bacterial abundance was relatively uniform throughout the water column, except for a peak in the 0.2 µm-Dec and 3.0 µm-Nov fractions of the 18 m samples that was more than 10% greater than average abundances. In ELB, the highest bacterial abundance was observed just below the ice cover (5 m). Abundance declined with increasing depth to 20 m and remained relatively constant between 20 m and 30 m depths. In WLB, bacterial abundance generally decreased with depth, an increase associated with the chemocline (10 m) was observed in all size fractions. In HOR, bacterial abundance increased from the surface to 8 m in the 0.2 µm fraction and 15 m in the 3 µm fraction and declined below these depths. The depth of abundance maximum is different between two size fractions in HOR, with an abundance peak at 7 m depth in 0.2 µm-Dec and at 15 m depth in 3.0 µm-Dec samples.

2.3.3. Distribution of bacterial phyla among lakes and depths

A total of 185,755 (on average 2,468 reads per sample ranging from 562 to 6,775) high-quality sequences were obtained from 78 samples. Good's coverage averaged 0.95 among the samples (ranging from 0.87 to 0.99). Sequences were clustered into 2,085 OTUs affiliated with 42 bacterial phyla. Variations were observed in bacterial diversity among the different lakes and

depths. Overall, Shannon diversity indices were higher in the 3.0 μm-filtered samples compared to the 0.2 μm-filtered samples (Paired-sample Wilcoxon signed-rank test, $P<0.01$) except for FRX. At FRX 15m depth, the bacterial diversity was much higher in 0.2 μm fractions than that in the 3.0 μm fractions fractions ($P<0.01$) (Table 2.3). Among 0.2 μm-filtered samples, the deepest layers of FRX (15 m depth) and WLB (30 m depth) had the highest bacterial diversity when compared to those of upper layers and no apparent trends were found in the other lakes (Table 2.3).

The distribution of major phyla differed by lake, depth and size fraction (Figure 2.3). *Bacteroidetes* and *Actinobacteria* were abundant across all depths and lakes. *Proteobacteria* was the next predominant phylum, most of which consisted of *Betaproteobacteria*. The two hypersaline lakes, ELB and WLB, had relatively similar phyla compositions, while the brackish or fresh water lakes (FRX, MIE and HOR) shared more common bacterial phyla. Phyla distribution also varied considerably with size fraction. Generally, *Actinobacteria* were more abundant in the 0.2 μm fractions compared to the 3.0 μm fractions at the equivalent depth. In contrast, *Planctomycetes*, *Verrucomicrobia* and *Cyanobacteria* were found more abundantly in the 3.0 μm fractions. Below the chemocline in FRX, candidate division WM88 (also classified as Hyd24-12 in SILVA taxonomy) was more abundant in 3.0 μm fractions. Candidate division JS1 (also known as OP9 or 'Atribacteria') was found more in the 0.2 μm fractions than in 3.0 μm fractions at the bottom layer

in FRX, and the relatively higher abundance of *Deltaproteobacteria* was observed in the 0.2 μm fractions at lower layers in MIE.

Each lake had distinct patterns of phyla by depth, especially the bottom depth. In FRX, *Betaproteobacteria*, *Planctomycetes* and candidate division WS5 decreased with increasing depth, while *Bacteroidetes* became more abundant from the surface of the lake to 9 m. The 0.2 μm fraction of the deepest layer (15 m depth) was dominated by various bacterial phyla, including *Proteobacteria* (mostly *Deltaproteobacteria,* 14.9% in Nov and 9.3% in Dec), *Tenericutes* (12.9% and 13.1%) and *Actinobacteria* (5.5% and 3.0%). In addition, candidate divisions such as JS1 (23.5% and 22.1%), WM88 (12.1% and 7.9%), SAR406 (5.4% and 11.4%), OD1 (5.0% and 8.5%) and Cloacamonas_p (4.3% and 3.1%) were also present in high abundance at this depth. Interestingly, candidate division WM88 constituted more than half (76.4% and 54.6%) of 3.0 μm fraction sequences at 15 m depth. In MIE, there was no apparent shift in phyla abundance down to the 15 m depth in 0.2 μm fraction, with over 80% of the total assemblages being dominated by *Bacteroidetes*, *Actinobacteria* and *Betaproteobacteria*. As expected, *Cyanobacteria,* most of which are filamentous, accounted for a large proportion of 3.0 μm fractions at 7-15 m depths (12.7-64.5% and 0.7-27.5% in relative abundance), whereas they were almost absent in 0.2 μm fractions at the same depths. At the bottom layer of MIE (18 m depth) the proportion of *Bacteroidetes* and *Actinobacteria* was relatively low while *Deltaproteobacteria* (28.3% and 38.1%) and *Chlorobi*

(18.2% and 9.1%) accounted for almost half of the total bacteria of the layer in 0.2 µm fractions. Also, *Alphaproteobacteria* and *Gammaproteobacteria* were markedly increased in 3.0 µm fractions. In ELB and WLB, *Bacteroidetes* and *Actinobacteria* were prominent at almost all depths. The distribution of bacterial phyla at 30 m was distinct in both ELB and WLB. The 3.0 µm fractions in ELB at this depth had a high proportion of *Gammaproteobacteria* (39.3% and 38.7%) and *Firmicutes* (44.6% and 48.3%); *Firmicutes* was also abundant at 30 m in WLB (43.4% and 30.7%). The abundance of *Bacteroidetes* at 30 m was dramatically lower in ELB (9.2% and 7.5%), while it was dominant in WLB (36.7% and 31.8%). HOR was also dominated by *Bacteroidetes* and *Actinobacteria* at all depths but *Bacteroidetes* continuously decreased in abundance down to 20 m and *Actinobacteria* were more abundant in two bottom depths (14 m and 20 m) than in the upper depths.

2.3.4. Bacterial differentiation between lakes and their relationship with physicochemical properties

There is a significant difference in bacterial community composition between different lakes in both size fractions (PERMANOVA, pseudo-$F_{4,37}$=3.15 and P<0.001 for 0.2 µm, pseudo-$F_{4,39}$=3.38 and P<0.001 for 3.0 µm). FRX and MIE had the most distinctive communities (PERMANOVA pairwise test, all P<0.01 but P=0.263 and P=0.087 with HOR, respectively), while WLB and ELB community compositions were more similar compared to the other lakes

(P=0.371). Depth was also one of the dominant factors for differentiating bacterial communities (P<0.001). The NMDS revealed that the bottom layers had the most distinctive assemblages in all lakes, whereas communities tended to converge in the surface layers when hierarchical clustering was overlaid onto the ordination space (Figure 2.4). Bacterial communities varied by different size fractions and sampling time, although not as large as the variation attributed to lake and depth. Sampling time did not significantly contribute to the community composition variation when modeled both alone and together with lake and depth variables (all P>0.05).

To determine which combination of environmental variables best explains the community variation, the most parsimonious model was selected using DISTLM and the result was graphically displayed using db-RDA. Best predictor variables varied when modeled on each individual lake (Figure 2.5). SO_4^{2-} was one of the best predictor variable sets in all lakes and in particular the single best predictor variable in MIE and WLB. The best predictor variable for FRX, ELB and HOR was BP, NO_3^- and DOC, respectively. Predictor variable sets differed by lake in multi-factor models. BP, Chl a, SO_4^{2-} and PPR were the major predictor variables in FRX. MIE community variation was mainly explained by depth, Chl a and SO_4^{2-}. NO_2^- was strongly associated with the bottom layer (30 m depth) of ELB, whereas deeper strata (17 m and 30 m depths) of WLB were positively correlated with NH_4^+. HOR community variation was best explained by SO_4^{2-} and pH.

2.3.5. Shared OTUs between lakes

Given the striking differences in community compositions among lakes and by depth, we investigated whether any of the taxa we detected were limited to a specific region of the lake indicating decreased niche width relative to the other bacterial taxa. Only 3% (62 OTUs) of the 2,085 OTUs were shared among the lakes when all data were combined (Figure 2.6). FRX harbored the largest proportion of unique OTUs (24.6%), whereas WLB had the least number of unique OTUs (8.6%). Certain bacterial lineages occurred exclusively at a particular depth of the lake. OTUs belonging to *Tenericutes* and *Deltaproteobacteria*, and several candidate divisions such as WM88, JS1 and SAR406 were almost exclusively found at the 15 m depth of FRX. Phylogenetic analysis based on the full-length 16S rRNA gene sequences revealed that these lineages are novel WM88 clades (FRX set2 and FRX 13), that are distinct from other known relatives. The JS1 clone sequence (FRX 39) was placed among lineages originated from subseafloor sediments (Figure 2.7 and Table 2.4). Sequences affiliated with genus *Ignavibacterium* of phylum *Chlorobi* were found only at 18 m depth of MIE (7.9-17.9% in relative abundance). *Virgibacillus* was found at 3.0 μm fraction of 30 m depth ELB (39.0% in Nov and 41.4% in Dec) and unknown Clostridiales genus (EU245980_g) occurred at 30 m depth WLB solely (11.0-22.8%). *Mariprofundus*-related OTUs of *Zetaproteobacteria* were present only at 17 and 30 m of WLB (0.7-1.6% and 3.1-3.7%, respectively).

2.4. Discussion

To our best knowledge, this is the first comprehensive comparison of bacterial communities based on high-throughput sequencing among the ice-covered lakes in McMurdo Dry Valleys, Antarctica. In previous studies, long-term research has largely focused on determination of the physicochemical characteristics and biological productivity of these unique lakes (Green and Lyons, 2009; Smith *et al*., 1993). Community composition studies have either focused on eukaryotes (Kong *et al*., 2014; Morgan-Kiss *et al*., 2016) or were conducted with limited samples and sequencing effort (Gordon *et al*.,2000; Vick-Majors *et al*., 2013). The present study has investigated the relationship between physicochemical properties and bacterial community composition using high-throughput sequencing techniques, which has revealed novel insights into the niche-specialization of bacterial lineages among depths and lakes.

It is not true for all cases but bacterial diversity was often higher in >3.0 μm size fractions than 0.2 μm equivalents across all samples. The reason for richness and diversity differences observed in the two fractions remains elusive, but a possible explanation is that particle-associated bacterial fraction can more easily obtain nutrients and survive in harsh aquatic environments (Berman, 1975). It is also possible that diverse bacterial lineages associated with microbial eukaryotes may contribute to the higher bacterial richness in larger size fractions (Li *et al*., 2016).

Contrary to the case of bacterial diversity, the relatively lower abundance in 16S rRNA gene copy numbers are observed in 3.0 μm fractions at upper layers, which may reflect the nature of sinking particles throughout the depth. However, vertical trends in 16S rRNA gene copies varied markedly by lake, depth and size fractions. It may be ascribed to variability in resource availability driven by local environmental conditions of each water column.

Bacteroidetes and *Actinobacteria* dominated the bacterial communities of all the lakes, a finding consistent with previous studies performed on Fryxell and Bonney (Vick-Majors *et al*., 2013; Glatz *et al*., 2006). Bacterial genera of these two phyla are similar to members found in other aquatic systems, and include freshwater and seawater lineages (Glöckner *et al*., 1999; Newton *et al*., 2011). *Betaproteobacteria* was also consistently found in high abundance in the deeper depths of all lakes. The half of assigned *Betaproteobacteria* (43.3% of total *Betaproteobacteria*) affiliated with genus *Polaromonas*. Members of *Polaromonas* display striking cold-adaptive characteristics as one of psychrophilic representatives of gram-negative bacteria (Feller and Gerday, 2003). They are ubiquitously found in cold environments and high-elevation environments such as glacial ice and periglacial soils across the globe (Darcy *et al*., 2011) and Antarctic lakes (Michaud *et al*., 2012). *Gammaproteobacteria* are dominant only in deeper layers of East and West Lobe Bonney, which may relate to the hypersaline environments of both lakes. Predominance of *Marinobacter* and *Halomonas* at

30 m depth of these lakes (22.0% and 67.5% of the total *Gammaproteobacteria*, respectively) indicates the existence of gammaproteobacterial lineages adapted to the high level of salinity present *in situ* (Bowman *et al*., 2000). In addition, other phyla such as *Cyanobacteria*, *Verrucomicrobia* and *Planctomycetes* were also frequently observed, although their relative abundance varied among lakes and depths. This is consistent with previous results that these groups are known to be typical and widespread members in freshwater and marine ecosystems (Fuerst, 1995; Schlesner, 1994).

Fine-scale taxonomic analysis revealed that certain bacterial taxa are confined to or absent from specific water layers, particularly at the deepest suboxic layer of each lake. In Fryxell, the deepest layer (15 m depth) was dominated by various uncultured bacterial groups belonging to candidate divisions JS1, WM88, and SAR406. Candidate division JS1 is commonly found in anoxic sedimentary systems, such as subsurface marine sediments (Parkes *et al*., 2007), methane hydrate-bearing sediments (Webster *et al*., 2007; Inagaki *et al*., 2006), Antarctic deep marine sediments (Carr *et al*., 2015) and sulfate-reducing sediments (Dhillon *et al*., 2003). Sediment enrichment and stable-isotope probing studies revealed some members of JS1 can utilize acetate and glucose under sulfate-reducing conditions (Webster *et al*., 2006; Webster *et al*., 2011). Abundance of JS1 in the bottom depth of Fryxell suggests certain JS1 members can be key bacterial representatives in anoxic water columns of brackish lakes as well as marine sediments.

WM88 members have been detected in various anoxic environments such as hydrothermal vents (Schauer *et al*., 2011), biofilms of sulfur-rich cave (Macalady *et al*., 2006), and hypersaline microbial mat (Harris *et al*., 2013). Two WM88 clones (FRX_set_2 and FRX_13) obtained in this study were matched most closely to sequences derived from a terrestrial mud volcano (98.6-98.8% 16S rRNA gene sequence similarities) (Cheng *et al*., 2012). These sequences were placed into a distinct clade divergent from other WM88 lineages in the phylogenetic tree. The identical 16S rRNA gene sequences, designated as 'unclassified OTU B1', were also found in high abundance in upper layers of Fryxell sediment (13.8-40.2% in relative abundance at the depth of 0-6 cm) and almost absent in lower sediment layers (Tang, 2009). Linking those sequences with metabolic functions is limited at this stage due to lack of functional information. Considering their dominance in the bottom waters and sediment of Fryxell, they must play a significant role in this isolated ecosystem. The relative abundance of WM88 markedly increased in the 3.0 μm size fraction compared to the 0.2 μm fraction, indicating this lineage may be present in particle-associated or aggregate forms. Another interesting point is that WM88 co-occur in this anoxic layer with other bacterial phyla such as SAR406, *Tenericutes*, Cloacamonas_p, OP3 and OP8. The exclusive presence of these various candidate divisions at the 15 m depth of Fryxell may link with unique biogeochemistry of anoxic environment of Fryxell. Further study is necessary to reveal why these lineages are exclusively found together at this depth and

how they have adapted to this unique environment.

There is a clear separation in *Firmicutes* lineages at the class level between 30 m in East and West Lobe Bonney. *Virgibacillus* (86.5% of the total *Bacilli*) belonging to class *Bacilli* dominated the bottom layer of East Lobe Bonney, whereas several genera belonging to class *Clostridia* were confined to the equivalent depth of West Lobe Bonney. The dominance of *Virgibacillus* in East Lobe Bonney is consistent with a previous finding, which reported that 35% of 16S rRNA gene clone library recovered from 25 m depth were closely related to *V. necropolis* and *V. salinus* (after re-matching reported sequences to those of recently updated species) (Glatz *et al*., 2006). Given the halotolerant or halophilic characteristics of cultured *Virgibacillus* spp., these lineages have specifically adapted to cold and halophilic environments of lower water layers of East Lobe Bonney (Glatz *et al*., 2006). The suboxic layers of East Lobe Bonney are characterized by extreme levels of nitrous oxide (N_2O) and a paucity of denitrification (Prisu *et al*., 1996). It was suggested that the high concentration of N_2O in East Lobe Bonney might be a 'biogeochemical relict' produced by past microbial activity on the basis of little association between contemporary active nitrifiers and high concentrations of N_2O (Priscu *et al*., 2008). Although there was no apparent evidence of association between N_2O and active microbes, our db-RDA result clearly showed that NO_2^- was an important factor in determining bacterial community structure at this depth.

The vast majority of *Firmicutes* from the bottom layer of West Lobe

Bonney are members of an unknown Clostridiales genus (EU245980_g) (76.5% of the total *Clostridia*) and to a much lesser extent *Halocella* (16.9%) and *Clostridium* (13.0%). Close relatives of the Clostridiales OTUs were frequently found in hypersaline microbial mat (Harris *et al*., 2013; Isenbarger *et al*., 2008). *Halocella* is known to be an obligately anaerobic halophile (Simankova *et al*., 1993) and many *Clostridium* species are commonly found in hypersaline environments (Oren, 2005). The *Clostridiales* OTUs were shown to be associated with the high NH_4^+ concentration (Caskey and Tiedje, 1980) at 17 and 30 m depth as revealed by db-RDA. Alternatively, these *Clostridiales* genera may gain energy for growth by Fe^{2+} oxidation coupled with denitrification or dissimilatory nitrate reduction under anoxic conditions (Weber *et al*., 2006). West Lobe Bonney's deep waters have high ferrous iron concentrations resulting from the iron-rich input of the adjacent Taylor Glacier's Blood Falls (Mikucki and Priscu, 2007). In addition, *Zetaproteobacteria* which were confined to 17 and 30 m depths, are reported to be iron oxidizers under microaerobic conditions (Emerson *et al*., 2007). Thus, ferrous ion oxidation may be an important process in West Lobe Bonney and its dependence on nitrate should be investigated in the future. However, it is unclear why distinct *Firmicutes* lineages dominate suboxic layers of East and West Lobe Bonney. These niche-differentiated populations could be formed as a result of long-term adaptation to a particular anoxic environment of each lake under different selection pressures.

In Miers, *Chlorobi* sequences belonging to the genus *Ignavibacterium* (98.2% of the total *Chlorobi*) were exclusively confined to 18 m depth; they were not found in other depths or lakes surveyed in this study. Phylum *Chlorobi* (green sulfur bacteria) are known for anaerobic photolithotrophs and inhabits the lower part of photic environments such as anoxic bottom layers of stratified lakes and microbial mats (Comeau *et al*., 2012; Iino *et al*., 2010). A recent study revealed that *Chlorobi* are more abundant in deeper anoxic microbial mats compared to those in top mats in Fryxell (Jungblut *et al*., 2016). However, given the lack of previous bacterial research in Miers, it is not clear that *Ignavibacterium* members found at the water column of 18 m depth are also dominant in anoxic microbial mats of equivalent depth. Class *Ignavibacteria* includes *Ignavibacterium* form a deeply branching clade in *Chlorobi* phylogeny. Chemoheterotrophy and the lack of apparent photosynthetic metabolism distinguish this class from other photosynthetic clades, which led to the proposal of a novel phylum (Iino *et al*., 2010; Podosokorskaya *et al*., 2013). In contrast to previous findings, bacterial production rate at this depth make it hard to preclude the possibility of phototropic lineages within this group. The metabolic potential of this lineage in Miers should be examined further by linking biogeochemical data with genomic evidence. Candidate division SR1 was found only in bottom layers of Miers and Fryxell, supporting its widespread occurrence in anoxic freshwater columns and sulfidogenic environments although certain members were also detected in oxic layers (Borrel *et al*., 2010).

There was little variation in community composition throughout the depths in Lake Hoare, with no niche-specific lineages being uniquely confined to each water column.

The bacterial composition of the upper layers of these lakes were relatively similar bacterial compared to the depths below the chemoclines. This similarity in community composition within the oxic layers of all lakes is likely due to similar environmental conditions formed at beneath the ice-cover and possible input from stream flow and the atmosphere to these lakes. Dieser and colleagues (Dieser *et al*., 2010) postulated that phylogenetic commonality among ice-embedded bacterial populations may result from environmental selection rather than other processes such as dispersal and seeding from a single origin material. However, it cannot exclude the possibility of dispersal effect as microbes are mixed via wind in the course of transporting aeolian material throughout the MDVs (Michaud *et al*, 2012; Sabacka *et al*., 2012). The relatively similar biogeochemical properties among the upper water columns of these lakes supports this contention and corresponds with the finding that similar bacterial communities are found within the ice covers of Lakes Fryxell, Miers, Hoare, East and West Lobe Bonney (Gordon *et al*., 2000).

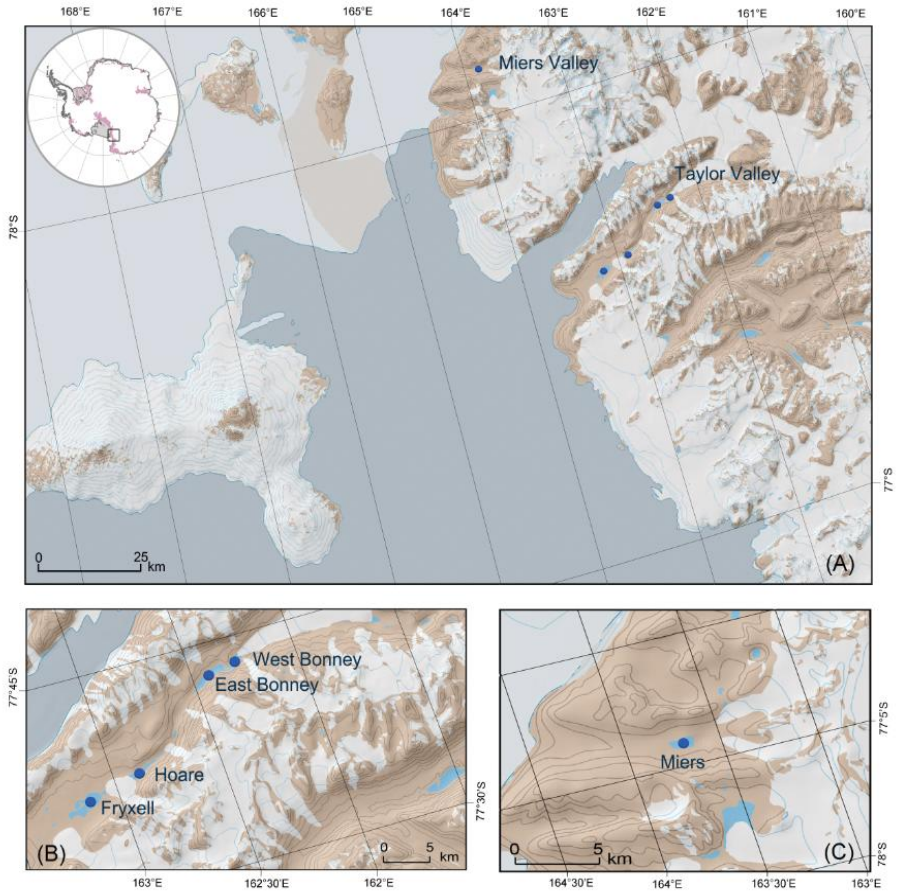

Figure 2.1. Map of sampling locations in (A) McMurdo Dry Valleys of Antarctica. (B) FRX, HOR, ELB and WLB are located in Taylor Valley, and (C) MIE in Miers Valley.

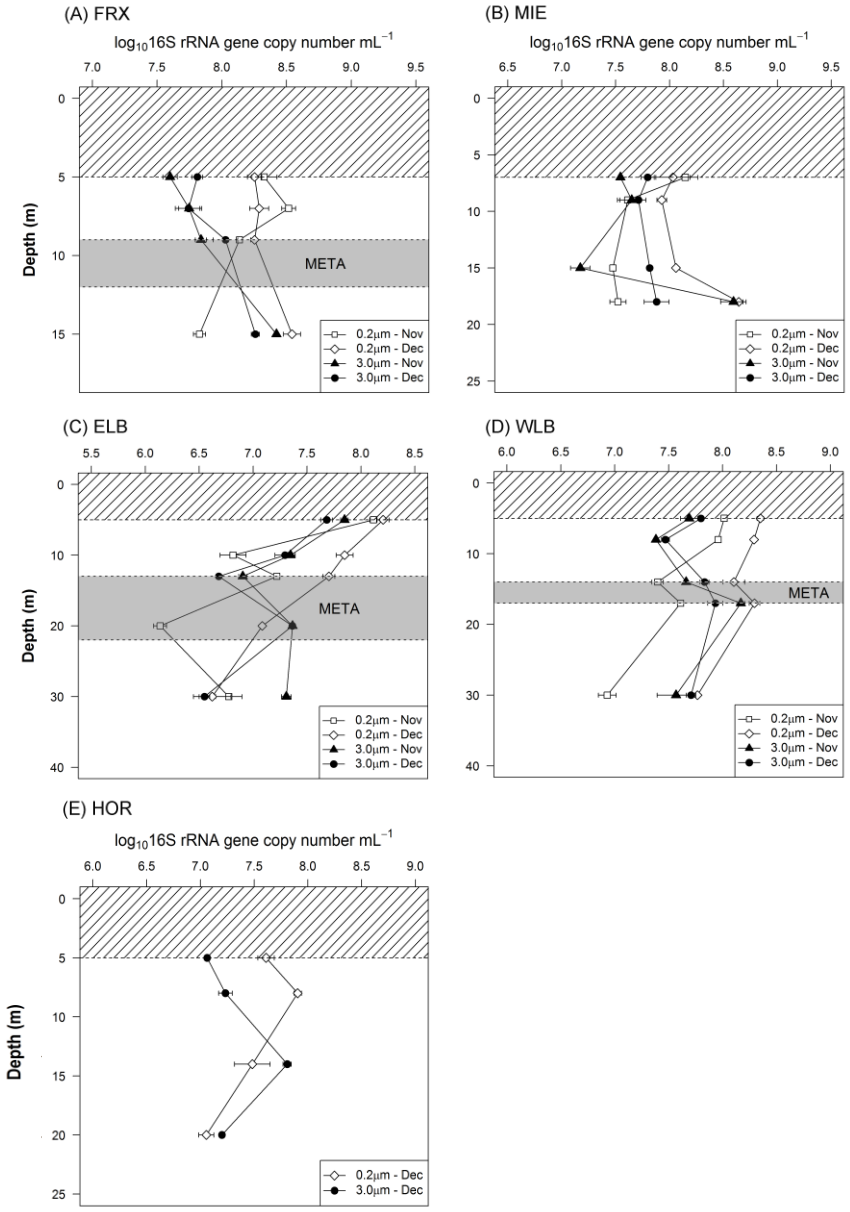

Figure 2.2. Depth profiles of bacterial 16S rRNA gene copy numbers in (A) FRX, (B) MIE, (C) ELB, (D) WLB and (E) HOR. Regions shaded with diagonal lines represent the permanent ice cover of each lake. Grey-shaded area indicate metalimnion (or chemocline).

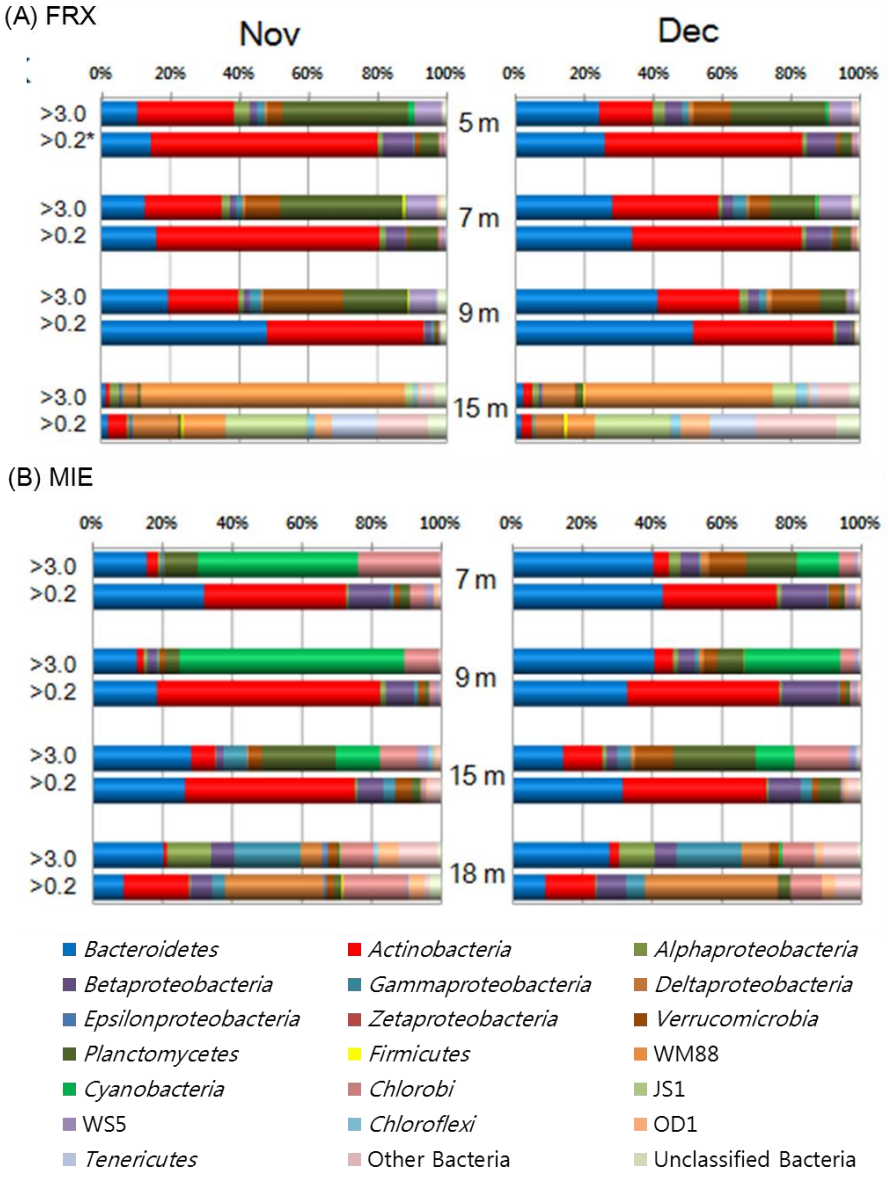

Figure 2.3. Bacterial phyla distribution differing by lake, depth and size fraction: (A) FRX and (B) MIE (1/3)

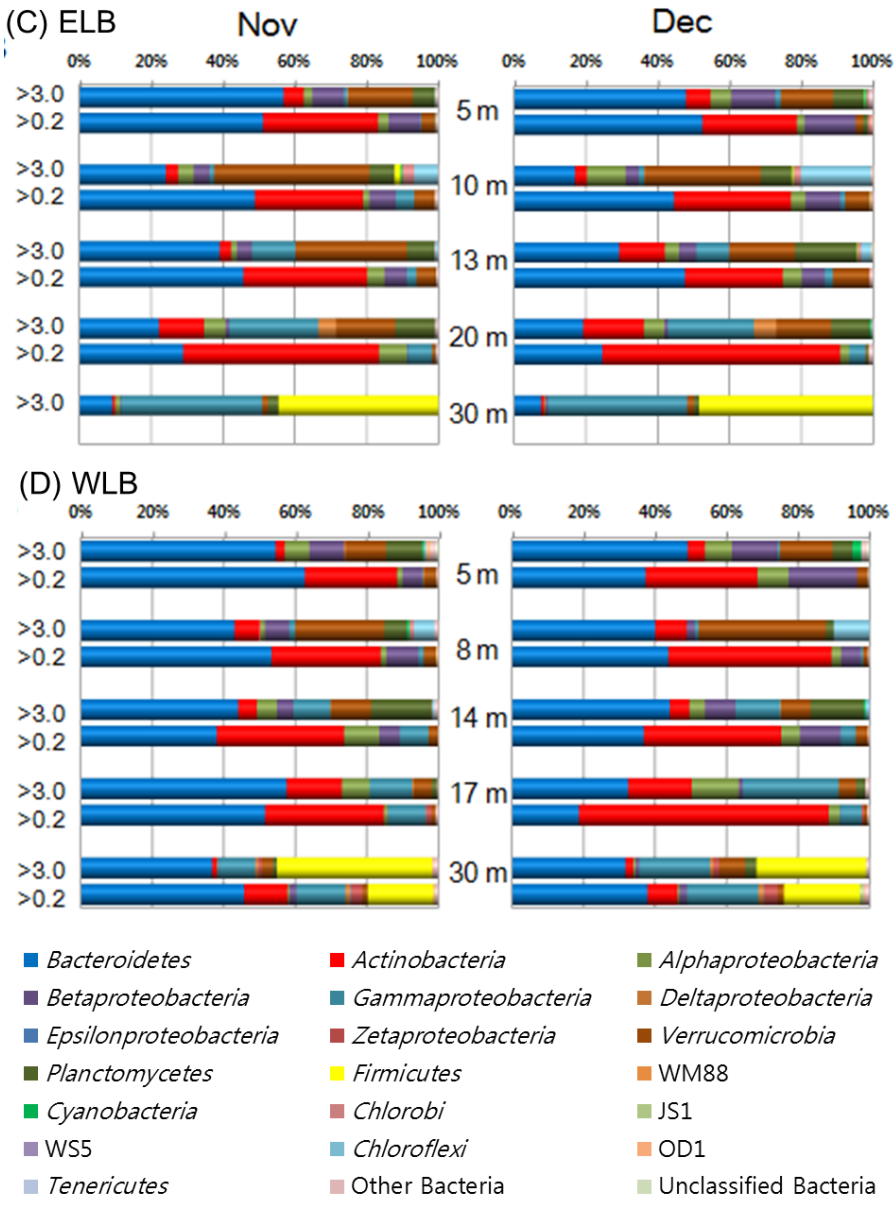

Figure 2.3. Bacterial phyla distribution differing by lake, depth and size fraction: (C) ELB and (D) WLB (2/3)

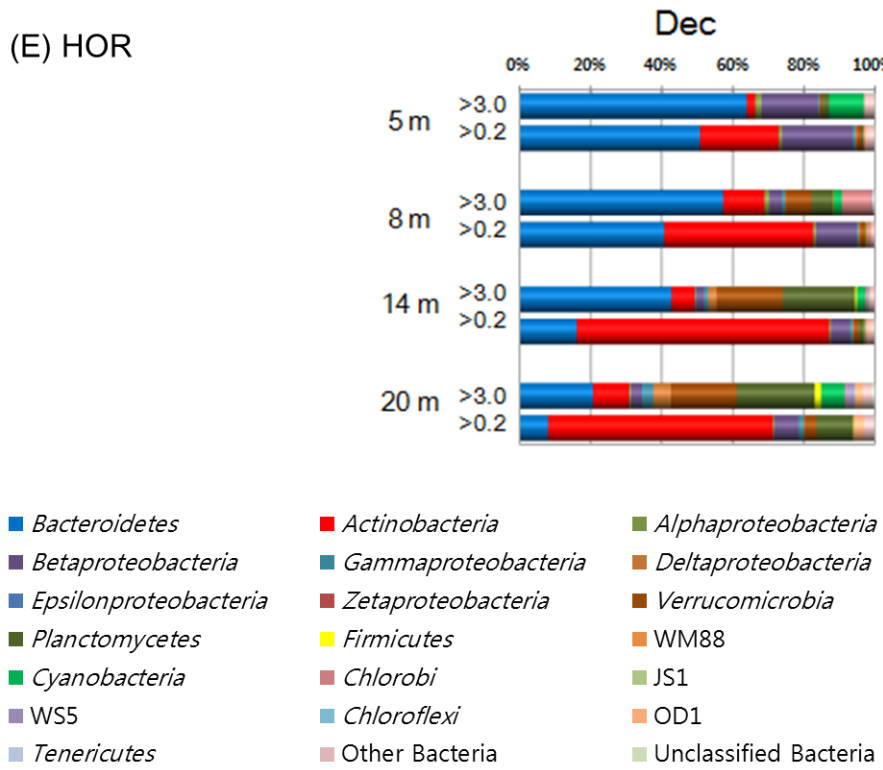

Figure 2.3. Bacterial phyla distribution differing by lake, depth and size fraction: (E) HOR (3/3). Other bacteria denote the group of relatively rare phyla with a relative abundance < 0.7 % in total reads among five lakes. Upper and lower bars denote the > 3.0 μm and the 0.2 to 3.0 μm fractions, respectively. The 0.2 μm fraction collected cells that passed through the 3.0 μm membrane filters.

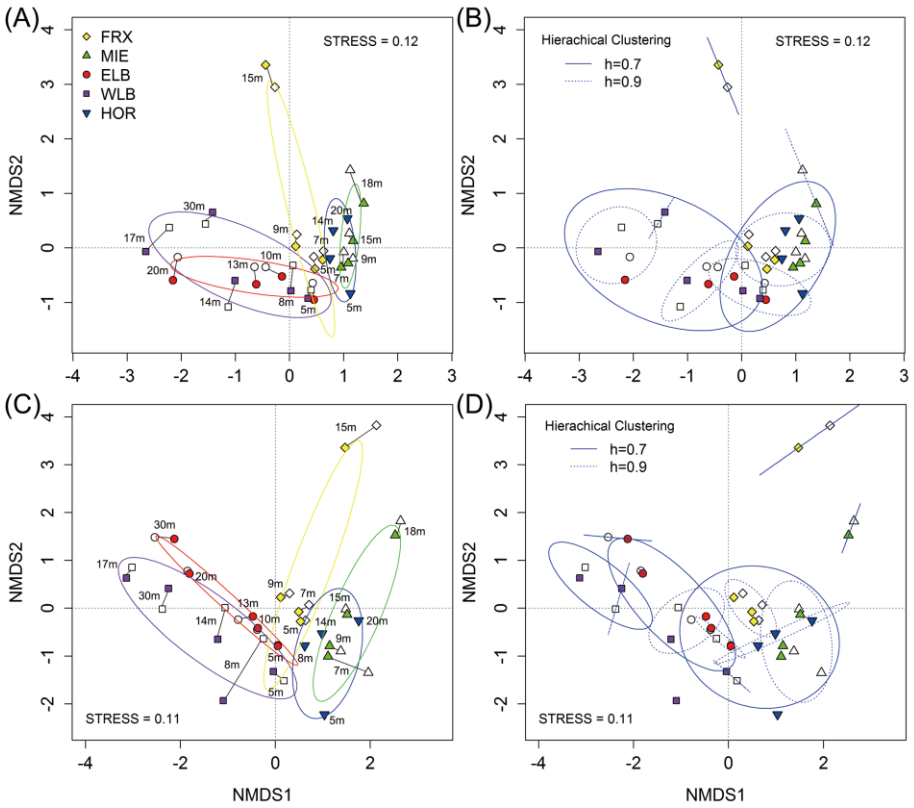

Figure 2.4. NMDS plot of OTU-level bacterial community composition: Panels (A) and (B) represent the 0.2 - 3.0 µm size fraction; Panels (C) and (D) represent the > 3.0 µm size fraction. OTU abundance matrices were used to calculate Bray-Curtis dissimilarities between samples. Hierachical clustering results were overlaid onto the ordination space in (A) and (C). Monthly samples at the same depth are linked by solid lines and samples from the same lake were grouped by ellipses with different colors in (B) and (D) to help visualize groupings between different lakes.

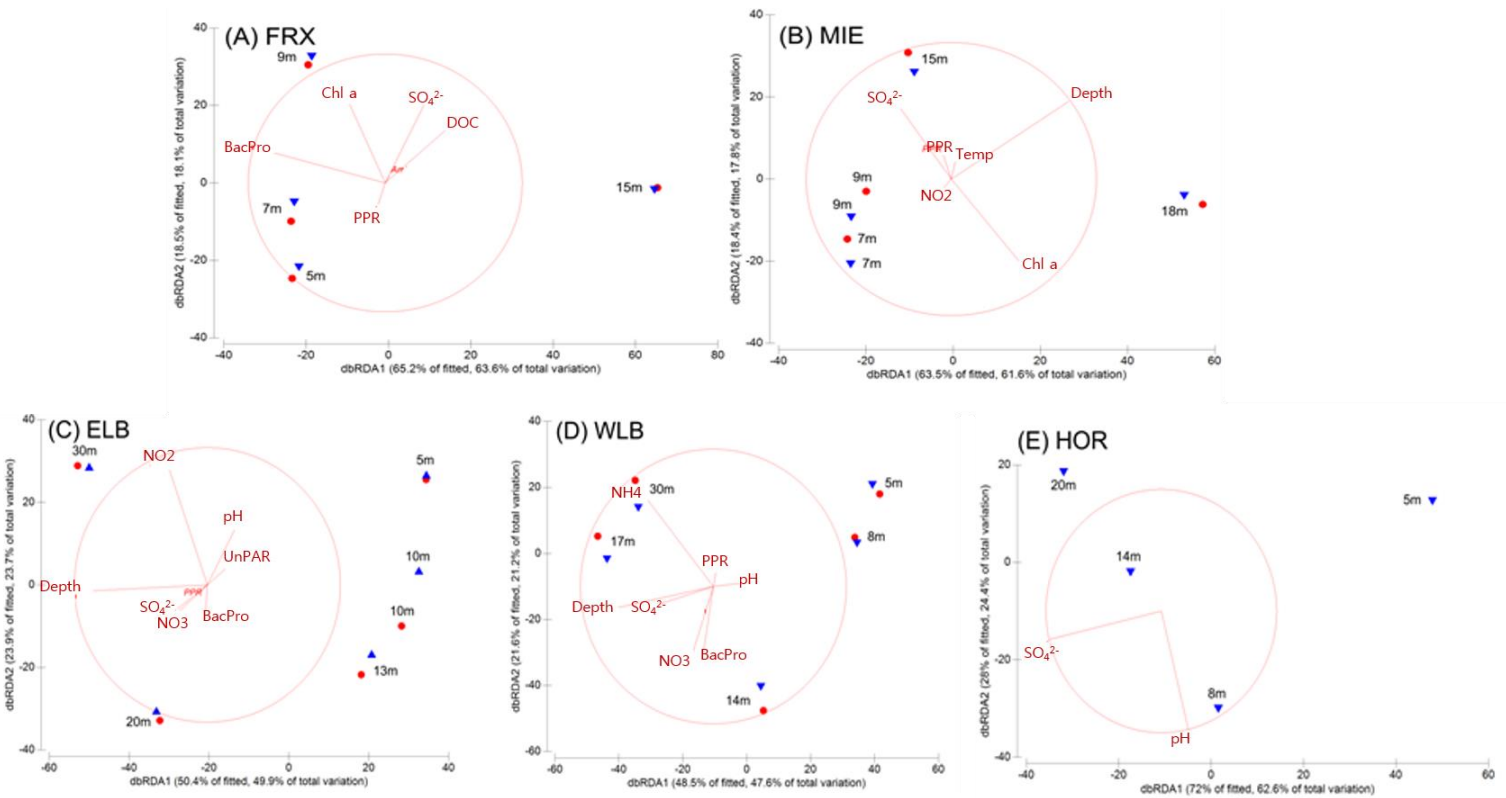
(A) FRX
9m
Chl a
SO_4^{2-}
DOC
BacPro
7m
PPR
5m
15m
dbRDA2 (18.5% of fitted, 18.1% of total variation)
dbRDA1 (65.2% of fitted, 63.6% of total variation)
(B) MIE
15m
SO_4^{2-}
Depth
PPR
Temp
9m
9m
NO2
7m
7m
Chl a
18m
dbRDA2 (18.4% of fitted, 17.8% of total variation)
dbRDA1 (63.5% of fitted, 61.6% of total variation)
(C) ELB
30m
NO2
5m
pH
UnPAR
10m
Depth
SO_4^{2-}
NO3
BacPro
10m
13m
20m
dbRDA2 (23.9% of fitted, 23.7% of total variation)
dbRDA1 (50.4% of fitted, 49.9% of total variation)
(D) WLB
NH4
30m
5m
17m
8m
PPR
pH
Depth
SO_4^{2-}
NO3
BacPro
14m
dbRDA2 (21.6% of fitted, 21.2% of total variation)
dbRDA1 (48.5% of fitted, 47.6% of total variation)
(E) HOR
20m
5m
14m
SO_4^{2-}
8m
pH
dbRDA2 (26% of fitted, 24.4% of total variation)
dbRDA1 (72% of fitted, 62.6% of total variation)

Figure 2.5. db-RDA plots of 'best' model selection by DISTLM for each number of environmental variables. (continued)

(A) FRX; there is multicollinearity between BP, DO and NH_4^+ ($|r| > 0.93$), and between SO_4^{2-}, depth, conductivity, DIC, DOC, Na, K, Mg, Ca and Cl ($|r| > 0.91$), (B) MIE; between depth, conductivity, DO, DIC, Mg, Ca, K and F ($|r| > 0.91$), between temperature, Na and Cl ($|r| > 0.97$), between PPR and AmbPAR ($r = 0.98$), and between SO_4^{2-} and BP ($r = -0.97$), (C) ELB; between SO_4^{2-}, conductivity, depth, DIC, NO_3^-, Na, K, Mg, Ca, Cl and UwPAR ($|r| > 0.91$), and between NO_2^-, DOC and Br ($|r| > 0.96$), (D) WLB; between SO_4^{2-}, conductivity, pH, DIC, depth, Na, K, Mg, Ca and Cl ($|r| > 0.93$), and between NH_4^+, DO and Br ($|r| > 0.91$), and (E) HOR; between SO_4^{2-}, conductivity, NO_2^-, DOC, DIC, Na, K, Mg, Ca, Cl and F ($|r| > 0.93$), and between depth, temperature, UwPAR and AmbPAR ($|r| > 0.91$).

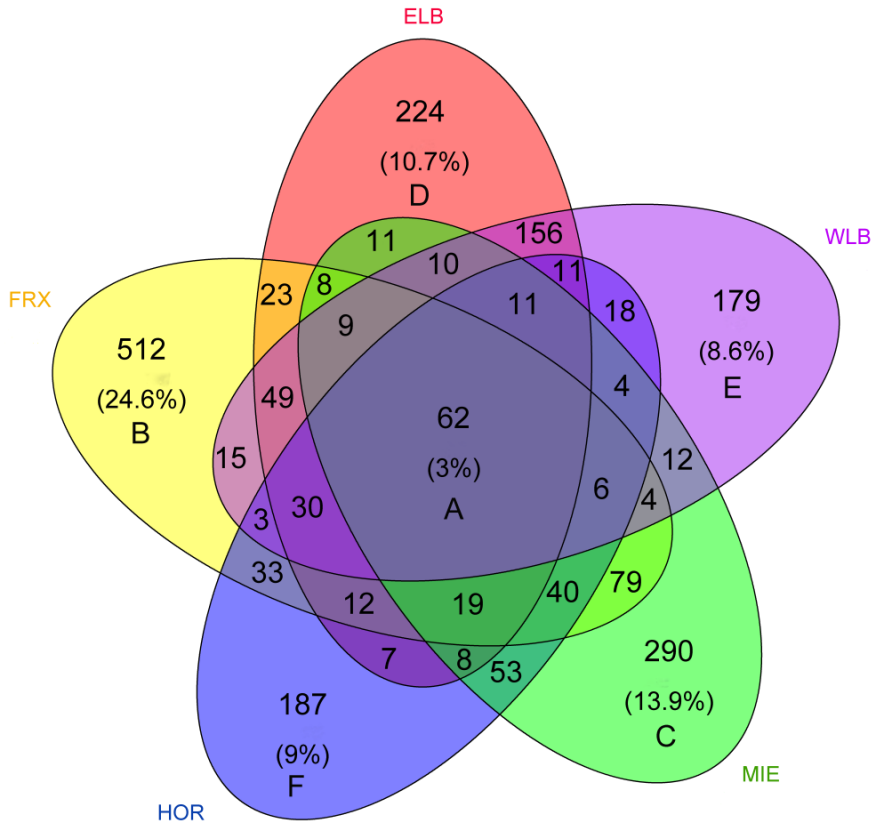

Figure 2.6. Venn diagram showing the overlap in bacterial OTUs among the five lakes. Numbers of OTUs represents all data combined (all depths, sampling times and size fractions in the lake).

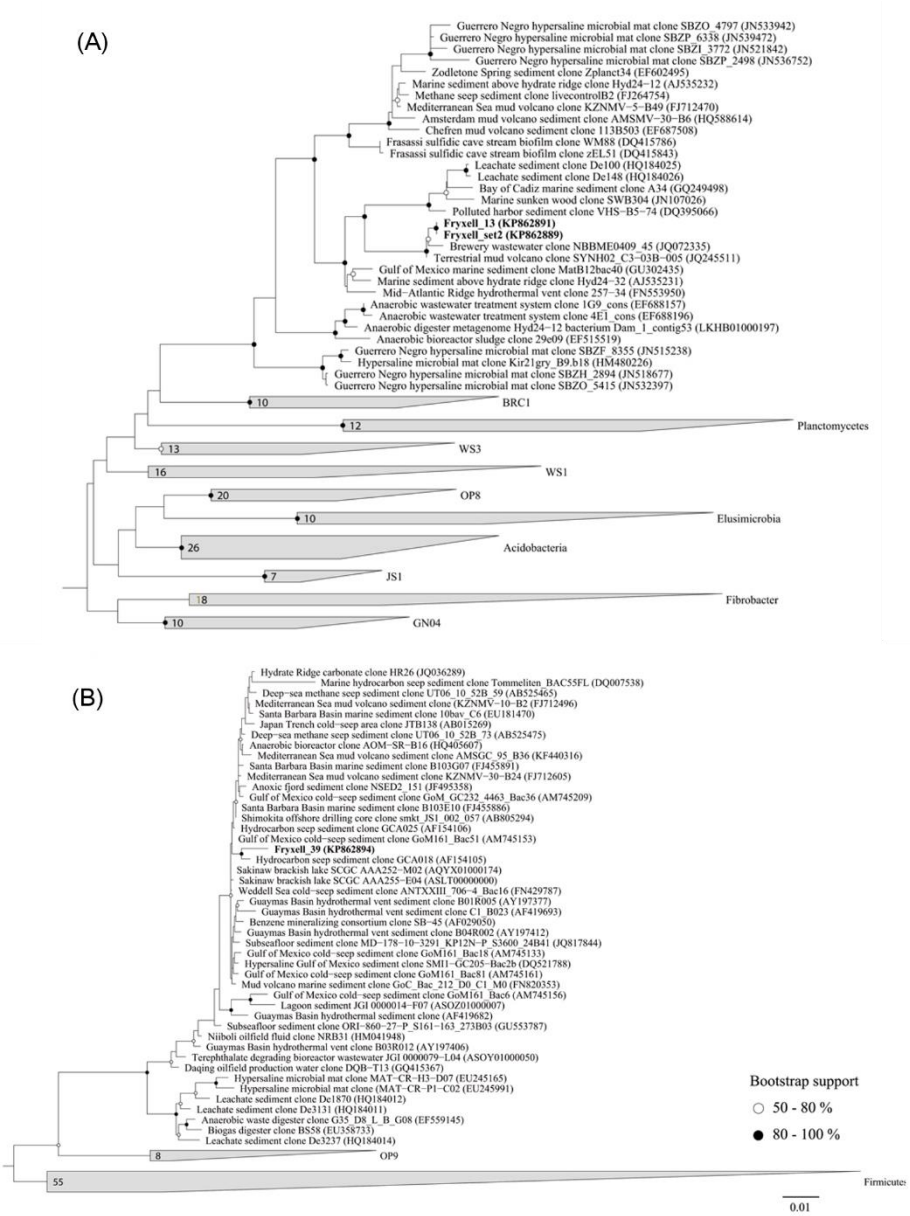

Figure 2.7. Phylogenetic positions of candidate division (A) WM88 and (B) JS1 lineages obtained from 15 m depth of FRX. The trees were constructed using maximum likelihood analysis and sequences belonging to phylum *Aquificae* were used as outgroups. Scale bar indicates 0.01 substitutions per nucleotide position.

Table 2.1. Physicochemical and biological characteristics at the sampling depths for each lake. (1/2)

Lake	Month	Depth (m)	Physical data					Biological data			Chemical data						
			Temp (ºC)	DO (mg O_2 L^{-1})	Cond. (mS cm^{-1})	UwPAR (umol phot m^{-2} s^{-1})	Amb. PAR (umol phot m^{-2} s^{-1})	BacPro (nM TDR)	Chl-a (μg L^{-1})	PPR (μg C L^{-1} d^{-1})	pH	DIC (mM)	DOC (mM)	SO_4^{2-} (mM)	NH_4^+ (µM)	NO_2^- (µM)	NO_3^- (µM)
FRX	Nov	5	1.31	25.511	0.53	14.52	1093	0.0158	2.55	2.24	8.34	3.10	0.15	0.29	0.05	0.17	0.07
		7	2.32	25.879	1.94	8.69	1091	0.0211	3.92	2.75	8.02	6.30	0.23	0.56	0.00	0.20	0.08
		9	2.70	28.372	3.24	3.53	1093	0.0321	7.69	1.77	7.78	11.70	0.40	1.02	0.04	0.15	0.10
		15	2.40	0.000	7.49	NA*	NA	0.0006	0.72	NA	7.38	42.90	1.42	1.84	335.72	0.48	1.09
	Dec	5	1.64	24.304	0.61	27.16	1269	0.0161	2.89	2.39	7.76	3.10	0.14	0.28	0.07	0.06	0.05
		7	2.54	24.904	2.18	15.91	1270	0.0299	4.23	2.23	7.71	5.30	0.20	0.47	0.00	0.05	0.01
		9	2.78	25.605	3.42	6.58	1272	0.0519	10.96	2.64	7.69	15.40	0.57	1.23	0.00	0.07	0.00
		15	2.39	0.000	7.60	0.02	1277	0.0003	0.72	NA	7.59	41.90	1.04	1.72	260.48	2.47	2.01
MIE	Nov	7	4.47	21.183	0.08	21.62	461	0.0048	1.48	2.66	8.74	1.00	0.05	0.05	0.42	0.05	0.30
		9	4.74	21.848	0.09	16.27	462	0.0009	1.28	3.45	8.81	1.00	0.05	0.05	0.20	0.04	0.13
		15	5.25	11.820	0.15	7.21	480	0.0038	1.31	3.48	8.30	1.80	0.05	0.05	5.07	0.06	0.10
		18	5.28	2.514	0.18	3.56	479	0.3363	5.96	3.66	7.89	2.30	0.08	0.03	14.82	0.07	0.06
	Dec	7	3.93	20.779	0.08	6.04	345	0.0173	0.97	0.68	8.13	0.90	0.02	0.04	NA	NA	NA
		9	4.70	22.310	0.09	4.77	348	0.0045	0.82	0.68	8.86	1.00	0.02	0.05	NA	NA	NA
		15	5.29	12.600	0.14	2.15	343	0.0023	1.01	0.76	8.53	1.80	0.02	0.05	NA	NA	NA
		18	5.46	2.948	0.16	1.14	340	0.1906	2.99	1.02	8.20	2.30	0.05	0.03	NA	NA	NA

Table 2.1. Physicochemical and biological characteristics at the sampling depths for each lake. (2/2)

Lake	Month	Depth (m)	Physical data					Biological data			Chemical data						
			Temp (ºC)	DO (mg O_2 L^{-1})	Cond. (mS cm^{-1})	UwPAR (umol phot m^{-2} s^{-1})	Amb. PAR (umol phot m^{-2} s^{-1})	BacPro (nM TDR)	Chl-a (μg L^{-1})	PPR (μg C L^{-1} d^{-1})	pH	DIC (mM)	DOC (mM)	SO_4^{2-} (mM)	NH_4^+ (µM)	NO_2^- (µM)	NO_3^- (µM)
ELB	Nov	5	1.97	26.934	1.33	17.25	1129	0.0038	2.01	1.35	7.61	1.00	0.05	0.96	1.12	0.33	12.51
		10	3.71	38.436	3.27	11.79	1130	0.0048	1.04	0.91	8.10	1.80	0.07	2.33	0.41	0.11	9.50
		13	4.42	39.031	7.00	8.73	1132	0.0049	0.92	0.83	7.77	2.30	0.06	2.73	1.66	0.18	14.82
		20	4.60	29.651	50.17	3.88	1134	0.0038	0.62	0.48	6.70	10.60	0.31	12.33	43.71	2.06	66.78
		30	2.47	0.347	113.48	1.21	1138	0.0022	0.35	NA	6.80	7.20	2.23	34.30	175.85	50.41	134.05
	Dec	5	2.01	29.824	1.29	14.21	1383	0.0174	3.90	0.96	8.39	1.00	0.05	0.94	0.71	0.22	11.13
		10	3.91	40.522	3.61	10.02	1388	0.0045	0.99	0.63	8.00	2.00	0.07	2.55	0.36	0.13	9.67
		13	4.50	42.280	8.63	7.62	1395	0.0054	0.97	0.60	7.75	2.60	0.07	2.82	2.01	0.20	16.91
		20	4.55	30.973	53.22	3.18	1429	0.0031	1.22	0.47	6.46	10.50	0.34	11.60	42.62	2.24	66.60
		30	2.79	0.318	113.98	0.76	1449	0.0004	0.20	NA	6.54	7.60	2.53	33.70	177.37	50.74	143.34
WLB	Nov	5	2.20	25.402	1.38	27.15	879	0.0066	1.74	1.28	8.19	0.90	0.05	0.79	0.86	0.31	13.78
		8	2.57	30.295	2.65	19.83	914	0.0038	1.10	1.21	8.00	1.50	0.05	1.64	0.45	0.14	8.42
		14	1.44	40.690	12.71	8.55	934	0.0195	2.06	2.32	7.41	4.20	0.12	4.45	0.28	0.29	12.22
		17	0.25	6.098	59.54	4.33	920	0.0033	2.59	1.62	6.13	38.50	0.74	38.50	149.55	1.24	14.01
		30	-2.77	0.000	78.04	0.41	778	0.0003	0.23	NA	5.89	68.30	1.47	47.70	241.26	0.96	0.32
	Dec	5	2.20	24.579	1.23	13.35	1202	0.0193	2.38	2.21	8.02	0.70	ND*	0.77	0.67	0.14	5.84
		8	2.50	31.710	2.55	9.60	1205	0.0028	1.01	0.96	7.72	1.60	0.02	1.89	0.27	0.14	7.06
		14	1.70	41.615	11.94	4.05	1211	0.0106	2.45	1.66	7.31	3.40	ND	4.04	0.72	0.39	13.82
		17	0.44	6.676	58.52	2.13	1213	0.0061	0.57	0.34	6.19	38.40	0.58	38.80	155.62	1.29	11.77
		30	-2.73	0.000	78.03	0.24	1224	0.0002	0.11	NA	5.74	64.00	1.17	46.20	254.69	0.86	0.25
HOR	Dec	5	0.64	16.212	0.30	6.24	1391	0.0273	0.30	NA	8.11	0.4	0.01	0.058	0.28	0.11	0.39
		8	0.53	36.789	0.51	3.84	1377	0.0081	1.82	NA	8.69	2.2	0.10	0.514	0.09	0.07	0.06
		14	0.41	35.719	0.63	1.38	1344	0.0065	3.45	NA	8.08	4.0	0.17	0.754	0.12	0.07	0.03
		20	0.21	16.790	0.70	0.51	1257	0.0022	1.11	NA	7.63	5.5	0.19	1.019	0.27	0.07	4.52

All samples were collected in 2012 to analyze pyrosequencing, physical, biological and chemical data except for nitrogen data in 2009-2010

Table 2.2. Ion chemistry at the sampling depths for each lake (1/2).

Lake	Collection date	Depth (m)	Ions							
			Li (mM)	Na (mM)	K (mM)	Mg (mM)	Ca (mM)	F (mM)	Cl (mM)	Br (mM)
FRX	Nov	5	ND*	8.48	0.61	1.09	1.53	0.03632	4.12	0.0054
		7	0.0041	11.40	0.68	1.40	1.32	0.04685	10.00	0.0139
		9	0.0059	23.80	1.32	2.79	2.11	ND	21.00	ND
		15	ND	90.00	4.12	11.50	3.47	0.31055	79.50	0.0756
	Dec	5	0.0009	4.70	0.33	0.55	0.89	ND	4.15	ND
		7	0.0029	9.13	0.55	1.04	1.18	ND	8.35	0.0094
		9	0.0067	32.20	1.71	3.46	2.43	ND	27.20	ND
		15	0.021	92.40	3.99	10.33	3.14	ND	77.60	0.104
MIE	Nov	7	ND	0.19	0.04	0.05	0.48	0.10	0.13	ND
		9	ND	0.22	0.04	0.06	0.49	0.12	0.15	ND
		15	0.0004	0.26	0.06	0.08	0.80	0.17	0.18	ND
		18	0.0004	0.27	0.07	0.10	0.98	0.19	0.17	ND
	Dec	7	0.0001	0.17	0.04	0.04	0.44	0.08	0.11	ND
		9	0.0001	0.21	0.04	0.05	0.52	0.10	0.14	ND
		15	0.0001	0.26	0.06	0.07	0.80	0.15	0.17	ND
		18	0.0003	0.26	0.07	0.09	1.01	0.19	0.17	ND

Table 2.2. Ion chemistry at the sampling depths for each lake (2/2).

Lake	Collection date	Depth (m)	Ions							
			Li (mM)	Na (mM)	K (mM)	Mg (mM)	Ca (mM)	F (mM)	Cl (mM)	Br (mM)
ELB	Nov	5	0.0043	7.74	0.25	1.21	1.19	ND	9.65	0.01
		10	ND	26.20	0.77	4.28	2.64	ND	31.60	0.06
		13	0.031	45.60	1.47	8.93	3.44	ND	62.60	0.14
		20	0.38	436.00	17.10	217.00	17.60	ND	948.00	3.84
		30	1.60	1727.00	72.70	1231.00	41.60	ND	4528.00	18.70
	Dec	5	0.0038	7.26	0.22	1.00	1.16	ND	8.91	0.02
		10	0.018	34.70	0.97	5.19	2.94	ND	43.00	0.07
		13	0.026	58.40	1.75	10.40	3.62	ND	78.40	0.22
		20	0.32	460.00	16.50	223.00	14.90	ND	916.00	3.15
		30	1.50	1948.00	68.80	1322.00	34.00	ND	4542.00	20.30
WLB	Nov	5	ND	6.39	0.19	0.89	0.98	ND	7.62	0.01
		8	ND	19.50	0.47	2.79	1.94	ND	23.10	0.04
		14	0.08	111.00	3.17	22.10	6.09	ND	156.00	0.41
		17	0.41	863.00	16.40	190.00	40.90	ND	1246.00	2.27
		30	0.59	1576.00	32.30	355.00	55.70	ND	2260.00	4.01
	Dec	5	0.00	5.92	0.17	0.74	0.89	0.31	6.97	0.01
		8	0.01	22.30	0.52	2.78	1.90	ND	27.30	0.00
		14	0.05	92.60	2.66	17.40	4.97	ND	133.00	0.00
		17	0.39	853.00	15.40	185.10	38.00	ND	1235.00	2.33
		30	0.88	1567.00	31.60	336.30	50.60	ND	2253.00	3.91
HOR	Dec	5	ND	0.318	0.037	0.056	0.129	0.00474	0.302	ND
		8	0.00072	3.023	0.317	0.539	0.803	0.03316	2.524	ND
		14	ND	5.263	0.524	0.926	1.282	0.06211	3.892	ND
		20	ND	6.481	0.627	1.152	1.664	0.08369	5.359	0.00394

All samples for Ion analysis were collected in 2012

* ND, Not detected

Table 2.3. Summary of pyrosequencing results and estimated diversity indices (1/3)

Lake	Date	Depth (m)	Cell size (µm)*	High quality reads	OTUs	OTUs†	Good's coverage	Shannon†	Simpson†	Inverse Simpson†	Chao†	ACE†	Coverage-based diversity‡		
													$q = 0$	$q = 1$	$q = 2$
FRX	Nov	5	>3.0	1853	112	82	0.94	3.27	0.08	12.79	104.14	114.79	69.69	23.13	11.04
			0.2 - 3.0	2418	125	75	0.94	2.80	0.18	5.70	120.00	186.05	69.98	13.45	4.73
		7	>3.0	1767	150	96	0.92	3.38	0.08	12.96	139.00	152.88	110.33	29.48	12.31
			0.2 - 3.0	1162	54	46	0.98	2.35	0.27	3.76	53.86	53.95	32.34	8.83	3.46
		9	>3.0	1401	125	92	0.93	3.53	0.06	17.19	138.87	165.09	90.70	32.52	16.76
			0.2 - 3.0	3940	142	58	0.95	2.24	0.23	4.37	83.00	92.97	57.51	9.36	4.41
		15	>3.0	1524	92	59	0.94	1.35	0.61	1.64	94.43	138.38	201.38	4.20	1.72
			0.2 - 3.0	3993	287	121	0.87	3.61	0.07	14.58	346.08	466.32	54.69	43.44	14.04
	Dec	5	>3.0	2374	156	99	0.94	3.67	0.05	18.43	127.33	134.70	99.84	37.10	17.18
			0.2 - 3.0	3105	121	62	0.95	2.62	0.19	5.24	109.25	124.25	57.27	14.74	5.53
		7	>3.0	2916	168	78	0.94	3.12	0.10	9.62	113.77	114.59	90.40	24.16	9.69
			0.2 - 3.0	1379	58	42	0.98	2.38	0.19	5.17	47.00	51.08	31.52	10.08	5.05
		9	>3.0	1814	156	99	0.92	3.65	0.05	19.52	165.00	195.56	113.06	37.20	16.97
			0.2 - 3.0	3911	121	55	0.94	2.28	0.23	4.37	179.00	206.94	43.47	9.32	4.32
		15	>3.0	6123	312	108	0.88	2.54	0.31	3.19	212.00	324.97	186.56	15.04	3.3
			0.2 - 3.0	892	120	107	0.93	3.72	0.06	16.98	141.17	154.61	106.45	39.87	16.00
MIE	Nov	7	>3.0	1532	46	34	0.98	1.99	0.25	3.93	39.50	46.28	20.61	6.62	3.83
			0.2 - 3.0	2786	130	64	0.95	3.07	0.08	12.59	104.60	166.01	65.73	22.31	12.37
		9	>3.0	2028	78	58	0.96	1.98	0.38	2.65	88.00	86.96	37.91	5.75	2.40
			0.2 - 3.0	1914	90	63	0.95	2.58	0.20	4.93	99.91	151.06	44.21	11.87	5.02
		15	>3.0	1593	119	71	0.96	3.30	0.06	16.22	92.23	95.46	76.59	27.77	15.80
			0.2 - 3.0	2142	108	74	0.95	3.02	0.13	7.43	92.06	104.10	60.42	19.74	7.72
		18	>3.0	1939	165	110	0.90	3.61	0.06	16.76	192.50	188.36	121.63	35.81	15.43
			0.2 - 3.0	2990	161	87	0.93	2.98	0.13	7.71	128.17	167.84	91.44	20.13	7.97
	Dec	7	>3.0	2751	130	74	0.94	3.41	0.05	19.62	109.43	170.41	69.20	29.80	18.47
			0.2 - 3.0	2422	102	59	0.95	2.88	0.09	10.89	88.25	126.06	46.60	18.96	11.75
		9	>3.0	2702	134	79	0.94	3.21	0.09	11.16	114.43	119.81	77.30	22.49	9.78
			0.2 - 3.0	2853	100	64	0.94	2.97	0.09	11.43	122.13	154.68	39.54	16.59	10.40
		15	>3.0	1508	114	82	0.94	3.24	0.07	13.60	119.06	120.09	75.90	25.27	13.91
			0.2 - 3.0	1744	80	62	0.95	3.05	0.08	13.15	99.80	158.60	44.97	19.19	12.08
		18	>3.0	2269	167	89	0.92	3.29	0.08	13.06	136.53	209.83	114.94	28.86	12.81
			0.2 - 3.0	2085	75	45	0.98	2.65	0.16	6.45	58.00	59.19	35.43	12.99	5.93

Table 2.3. Summary of pyrosequencing results and estimated diversity indices (2/3)

Lake	Date	Depth (m)	Cell size (μm)*	High quality reads	OTUs	OTUs†	Good's coverage	Shannon†	Simpson†	Inverse Simpson†	Chao†	ACE†	Coverage-based diversity‡		
													$q = 0$	$q = 1$	$q = 2$
ELB	Nov	5	>3.0	2380	115	71	0.96	3.23	0.08	13.10	83.83	90.80	67.79	26.82	14.13
			0.2 - 3.0	3237	104	58	0.96	2.95	0.09	11.03	86.11	84.50	41.49	16.64	9.57
		10	>3.0	2219	129	80	0.95	3.45	0.06	17.88	99.89	112.34	78.83	29.23	15.42
			0.2 - 3.0	4283	125	62	0.95	2.98	0.09	10.72	94.50	125.84	53.13	18.22	9.97
		13	>3.0	2496	116	67	0.95	2.87	0.12	8.06	103.11	92.10	58.84	16.63	7.85
			0.2 - 3.0	6524	139	56	0.96	2.64	0.13	7.42	83.60	119.02	43.77	13.42	7.36
		20	>3.0	3374	196	107	0.91	3.84	0.04	27.11	182.20	213.48	119.97	48.60	25.68
			0.2 - 3.0	3862	104	54	0.96	2.24	0.27	3.71	71.27	88.97	47.63	9.63	3.75
		30	>3.0	2294	93	66	0.96	2.91	0.11	8.70	89.00	91.46	50.70	16.34	8.35
	Dec	5	>3.0	2673	144	85	0.95	3.68	0.04	23.09	108.40	111.63	80.51	40.29	24.13
			0.2 - 3.0	1148	43	36	0.98	2.74	0.10	9.77	51.00	47.05	24.36	13.75	8.72
		10	>3.0	2996	139	83	0.94	3.39	0.07	15.11	168.00	125.88	76.21	26.46	13.30
			0.2 - 3.0	2490	99	65	0.95	3.33	0.05	18.52	111.43	135.29	51.43	24.97	15.88
		13	>3.0	1787	124	91	0.94	3.55	0.05	18.41	118.56	123.39	87.11	34.79	18.43
			0.2 - 3.0	2615	78	50	0.96	2.65	0.12	8.04	65.83	99.93	30.92	12.73	8.01
		20	>3.0	2782	193	107	0.91	3.73	0.05	21.41	213.00	227.67	125.90	45.22	21.59
			0.2 - 3.0	830	50	42	0.97	1.86	0.34	2.96	54.00	59.34	28.50	6.16	2.95
		30	>3.0	6775	158	66	0.95	2.77	0.13	7.55	109.50	107.17	52.41	14.47	7.41
WLB	Nov	5	>3.0	573	66	66	0.96	3.31	0.07	14.56	93.14	83.36	53.51	25.54	13.87
			0.2 - 3.0	3496	110	60	0.94	2.67	0.14	6.95	118.13	109.09	41.70	13.15	6.14
		8	>3.0	1270	109	77	0.96	3.67	0.04	27.84	95.07	97.69	76.51	39.68	26.71
			0.2 - 3.0	5227	137	68	0.95	3.20	0.08	13.18	93.00	119.53	48.54	22.32	13.3
		14	>3.0	2017	127	85	0.93	3.30	0.08	12.20	136.23	131.11	79.31	27.13	12.82
			0.2 - 3.0	2041	41	30	0.99	2.36	0.15	6.87	31.88	34.45	16.11	8.63	5.90
		17	>3.0	1131	88	69	0.95	2.96	0.13	7.84	94.00	114.56	57.84	16.68	7.07
			0.2 - 3.0	5178	125	55	0.95	2.51	0.16	6.35	125.20	126.44	40.16	11.96	6.32
		30	>3.0	799	81	75	0.95	3.35	0.06	15.99	102.00	105.67	64.84	28.03	15.88
			0.2 - 3.0	3628	159	90	0.94	3.57	0.06	18.12	114.80	124.41	92.66	34.93	17.36
	Dec	5	0.2 - 3.0	841	47	42	0.98	2.93	0.08	12.67	53.00	52.56	29.38	16.78	11.59
		8	0.2 - 3.0	743	47	44	0.99	2.88	0.09	10.56	45.67	47.02	33.82	15.55	9.4
		14	>3.0	562	72	72	0.95	3.30	0.07	14.26	115.88	102.32	62.24	25.99	13.78
			0.2 - 3.0	2586	71	40	0.98	2.46	0.15	6.77	51.38	55.82	25.29	10.34	6.19
		17	0.2 - 3.0	2440	67	37	0.98	1.63	0.44	2.29	43.60	48.52	26.48	5.07	2.33
		30	>3.0	900	134	116	0.91	3.95	0.03	31.06	185.79	181.81	134.00	53.12	29.70
			0.2 - 3.0	2989	172	97	0.93	3.69	0.04	22.24	148.25	177.98	106.34	41.26	21.31

Table 2.3. Summary of pyrosequencing results and estimated diversity indices (3/3)

Lake	Date	Depth (m)	Cell size (μm)*	High quality reads	OTUs	OTUs†	Good's coverage	Shannon†	Simpson†	Inverse Simpson†	Chao†	ACE†	Coverage-based diversity‡		
													$q = 0$	$q = 1$	$q = 2$
HOR	Dec	5	>3.0	2339	176	117	0.91	3.88	0.04	25.45	168.04	177.28	130.32	54.41	28.28
			0.2 - 3.0	3362	188	83	0.93	3.15	0.10	9.70	126.94	170.30	111.30	24.19	9.00
		8	>3.0	999	90	76	0.96	3.55	0.05	21.11	92.50	96.84	68.22	33.78	20.03
			0.2 - 3.0	4538	140	63	0.95	2.90	0.11	9.51	94.91	92.52	53.42	17.82	9.76
		14	>3.0	700	88	82	0.95	3.48	0.05	18.48	115.46	114.50	75.83	32.04	18.00
			0.2 - 3.0	2086	99	48	0.96	1.96	0.34	2.93	78.00	71.13	49.61	8.03	3.15
		20	>3.0	923	104	91	0.94	3.64	0.05	21.98	133.00	134.58	84.48	37.09	20.85
			0.2 - 3.0	3168	135	75	0.94	2.49	0.26	3.85	124.58	115.45	71.82	12.36	4.01

* Cell size is larger than 3.0 μm and between 3.0 μm and 0.2 μm.

† Diversity indices were estimated at the equal library size (n=562) per sample.

‡ Diversity was estimated at the equal sample coverage (0.943) per sample for Hill numbers of $q = 0$ (species richness), $q = 1$ (the exponential of Shannon's entropy index), and $q = 2$ (the inverse of Simpson's concentration index).

Table 2.4. BLAST results of candidate division WM88 against Genbank database (1/2)

Accession	Description	Identity	Isolation source
JQ072335	Uncultured bacterium clone NBBME0409_45	98%	Brewery wastewater
JQ245511	Uncultured bacterium clone SYNH02_C3-03B-005	99%	Mud volcano
FN553950	Uncultured sediment bacterium clone 257-34	92%	Logatchev hydrothermal vent
AJ535231	Uncultured bacterium clone Hyd24-32	91%	Marine sediment above hydrate ridge
GU302435	Uncultured bacterium clone MatB12bac40	91%	Marine sediments from Mississippi Canyon
HQ184026	Uncultured bacterium clone De148	91%	Leachate sediment
HQ184025	Uncultured bacterium clone De100	91%	Leachate sediment
GQ249498	Uncultured bacterium clone A34	91%	Marine sediment
DQ395066	Uncultured bacterium clone VHS-B5-74	90%	Harbor sediment
JN107026	Uncultured bacterium clone SWB304	90%	Marine sunken wood
EF602495	Uncultured bacterium clone Zplanct34	90%	Zodletone spring source sediments
JN518677	Uncultured organism clone SBZH_2894	89%	Uerrero Negro hypersaline Mat
DQ415786	Uncultured bacterium clone WM88	89%	Frasassi sulfidic cave stream biofilm
DQ415843	Uncultured bacterium clone zEL51	89%	Frasassi sulfidic cave stream biofilm
JN532397	Uncultured organism clone SBZO_5415	89%	Guerrero Negro hypersaline Mat
JN515238	Uncultured organism clone SBZF_8355	89%	Guerrero Negro hypersaline Mat
HQ588614	Uncultured bacterium clone AMSMV-30-B6	89%	Amsterdam mud volcano sediment
EF515519	Uncultured bacterium clone 29e09	89%	Anaerobic bioreactor sludge
LKHB01000197	Candidate division Hyd24-12 bacterium Dam_1 contig53	89%	Enrichment reactor seeded with activated sludge
FJ264754	Uncultured bacterium clone livecontrolB2	88%	Methane seep sediment in El River basin
EF688157	Uncultured bacterium clone 1G9_cons	88%	Anaerobic wastewater treatment system
JN521842	Uncultured organism clone SBZI_3772	88%	Guerrero Negro hypersaline Mat
HM480226	Uncultured bacterium clone Kir21gry B9.b18	88%	Hypersaline microbial mat
EF687508	Uncultured bacterium clone 113B503	88%	Mud volcano
JN539472	Uncultured organism clone SBZP_6338	88%	Guerrero Negro hypersaline Mat
AJ535232	Uncultured bacterium partial clone Hyd24-12	88%	Marine sediment above hydrate ridge
FJ712470	Uncultured bacterium clone KZNMV-5-B49	88%	Kazan mud volcano
EF688196	Uncultured bacterium clone 4E1_cons	87%	Anaerobic wastewater treatment system
JN533942	Uncultured organism clone SBZO_4797	87%	Guerrero Negro hypersaline Mat
JN536752	Uncultured organism clone SBZP_2498	87%	Guerrero Negro hypersaline Mat

Table 2.4. BLAST results of candidate division JS1 against Genbank database (2/2)

Accession	Description	Identity	Isolation source
AM745153	Uncultured candidate division JS1 bacterium clone GoM161_Bac51	99%	Marine sediments
AQYX01000174	Atribacteria bacterium SCGC AAA252-M02 OP9_1DRAFT_contig_9.10_C	99%	Sakinaw Lake
ASLT01000000	Atribacteria bacterium SCGC AAA255-E04 A255E4DRAFT_contig_0_0.1_C	99%	Sakinaw Lake brackish water
FN429787	Uncultured candidate division JS1 bacterium clone ANTXXIII_706-4_Bac16	99%	Marine sediments
DQ521788	Uncultured bacterium clone SMI1-GC205-Bac2b	99%	Gulf of Mexico sediments
JQ817844	Uncultured bacterium clone MD-178-10-3291_KP12N-P_S3600_24B41	99%	Subseafloor sediment at the Kaoping Canyon
AF154106	Uncultured bacterium clone B103E10	99%	Marine sediment
AM745133	Uncultured candidate division JS1 bacterium clone GoM161_Bac18	98%	Marine sediments
FN820353	Uncultured bacterium clone GoC_Bac_212_D0_C1_M0	98%	Marine sediment of mud volcano
FJ455886	Uncultured bacterium clone B103E10	98%	Marine sediments
JF495358	Uncultured bacterium clone NSED2_151	98%	Anoxic fjord sediment
AF419693	Uncultured bacterium clone C1_B023	98%	Guaymas Basin hydrothermal vent sediments
AY197377	Uncultured bacterium clone B01R005	98%	Guaymas Basin hydrothermal vent sediments
AY197412	Uncultured bacterium clone B04R002	98%	Basin hydrothermal vent sediments
AF154105	Uncultured bacterium clone GCA018	98%	Hydrocarbon seep sediment
JQ036289	Uncultured bacterium clone HR26	98%	Hydrate Ridge carbonate 2518
AM745161	Uncultured candidate division JS1 bacterium clone GoM161_Bac81	98%	Marine sediments
FJ712605	Uncultured bacterium clone KZNMV-30-B24	98%	Kazan Mud Volcano
AB805294	Uncultured bacterium clone smkt_JS1_002_057	98%	Ocean drilling core sample
AF029050	Benzene mineralizing consortium clone SB-45	98%	Guaymas Basin sediments
AM745209	Uncultured candidate division JS1 bacterium clone GoM_GC232_4463_Bac36	98%	Marine sediments
HQ405607	Bacterium enrichment culture clone AOM-SR-B16	98%	Enrichment mediating the anaerobic oxidation of methane
FJ712496	Uncultured bacterium clone KZNMV-10-B2	98%	Kazan mud volcano
KF440316	Uncultured bacterium clone AMSGC_95_B36	98%	Amsterdam mud volcano
FJ455891	Uncultured bacterium clone B103G07	97%	Marine sediments
EU181470	Uncultured bacterium clone 10bav_C6_ARB	97%	Marine sediments
AB525465	Uncultured bacterium clone UT06_10_52B_59	96%	Deep-sea methane seep sediment off Joetsu
AB525475	Uncultured bacterium clone UT06_10_52B_73	96%	Deep-sea methane seep sediment off Joetsu
GU553787	Uncultured bacterium clone ORI-860-27-P_S161-163_273B03	96%	Subseafloor sediment at the Yung-An Ridge
HM041948	Uncultured candidate division JS1 bacterium clone NRB31	95%	Fluid from Niiboli oilfield
AF419682	Uncultured bacterium clone CS_B013	95%	Hydrothermal sediments in the Guaymas Basin
AB015269	Uncultured candidate division JS1 bacterium clone JTB138	95%	Deepest cold-seep area of the Japan Trench
AY197406	Uncultured bacterium clone B03R012	94%	Guaymas Basin hydrothermal vent sediments
AM745156	Uncultured candidate division JS1 bacterium clone GoM161_Bac6	94%	Marine sediments
GQ415367	Uncultured bacterium clone DQB-T13	93%	Daqing oilfield production water
ASOY01000050	Atribacteria bacterium JGI 0000079-L04 J0000079L04DRAFT_contig_48_0.50_C	93%	TA biofilm SAG 231
ASOZ01000007	Atribacteria bacterium JGI 0000014-F07 J0000014F07DRAFT_contig_5_0.7_C	93%	Etoliko laggon SAG 227
DQ007538	Uncultured bacterium clone Tommeliten_BAC55FL	92%	Marine hydrocarbon seep sediment
HQ184014	Uncultured bacterium clone De3237	90%	Leachate sediment
EU245165	Uncultured organism clone MAT-CR-H3-D07	90%	Hypersaline microbial mat
EF559145	Uncultured bacterium clone G35_D8_L_B_G08	89%	Mesophilic anaerobic digester
EU245991	Uncultured organism clone MAT-CR-P1-C02	89%	Hypersaline microbial mat
EU358733	Uncultured bacterium clone BS58	89%	Mesophilic biogas digester treating pig manure
HQ184011	Uncultured bacterium clone De3131	89%	Leachate sediment
HQ184012	Uncultured bacterium clone De1870	89%	Leachate sediment

CHAPTER 3.

Culture-independent approach:

Insights into ecological function of sulfate-reducing bacteria using metagenomic analysis in ice-covered Lake Fryxell

3.1. Introduction

Dissimilatory sulfate reduction is an important process in the organic matter cycling as one of the oldest types of biological energy conservation in the aquatic ecosystem (Shen *et al.*, 2001). Over 50% of organic carbon is mineralized by sulfate-reducing prokaryotes in marine sediments (Jørgensen, 1982). In the anaerobic condition, sulfate-reducing bacteria show higher affinity to substrates (Widdel, 1988), and organic compounds are degraded to CH_4, CO_2 and H_2S (Dar *et al.*, 2008). Sulfate-reducing bacteria (SRB) use sulfate as a terminal electron acceptor, and produce sulfide as a byproduct. The dissimilatory process is mediated by three key enzymes of SRB, which serially catalyze reduction of sulfate, sulfite to sulfide such as ATP sulfurylase (Sat), adenosine-5'-phosphosulfate reductase (Apr), and dissimilatory sulfite reductase (Dsr). In particular, Dsr have been found in all SRB, which play an important role in last step of catalyzing reduction of sulfate to sulfide. For these reasons, Dsr genes have been applied to assess a tree of life for SRB along with 16S rRNA gene from diverse environment samples (Loy *et al.*, 2002, Leloup *et al.*, 2006, Moreau *et al.*, 2010).

SRB has been ubiquitously found in many different environments such as hydrothermal vent (Jeanthon *et al.*, 2002), salt marsh (Bahr *et al.*, 2005), hypersaline microbial mats (Risatti *et al.*, 1994), marine sediment (Mußmann *et al.*, 2005) and freshwater sediments (Sass *et al.*, 1998). The diversity and dynamics of SRB have been studied by both culture-dependent and -

independent, molecular techniques such as DGGE and fluorescence *in situ* hybridization (FISH) (Rabus *et al.*, 1996, Dar *et al.*, 2008). In spite of their diversity, SRB is still poorly understood in extreme environments such as Antarctica due to limited access.

Lake Fryxell (FRX) is permanently covered with the ice about 4 m in thickness, which is located in McMurdo Dry Valleys (MDVs), East Antarctica. FRX demonstrates geochemically stratified and stable water columns because the ice cover prevents wind turbulence and vertical mixing. Interestingly, the bottom depth of FRX has characteristics of anoxic, brackish, and high concentration of sulfide. The concentration of sulfate increased with depth and peaked at 15 m depth with 1.84 mM, then decreased to about 0.1 mM at 18 m depth (Tang 2009, Kwon *et al*., 2017). Preceding study elucidated the existence of not only a variety of bacteria but also candidate phyla in the bottom of FRX, which were WM88 and JS1 that were predominantly distributed about over 70% and 30% onto 3.0 and 0.2 μm filters, respectively (Kwon *et al.*, 2017), even in extreme environment. In previous studies, sulfate reduction was actively detected (Sattley & Madigan, 2010), and the diversity of SRB was investigated by anaerobic cultivation, clone libraries and functional gene analysis in FRX (Karr et al. 2005, Tang 2009). However, according to small portion of SRB were cultivated, it is impenetrable to understand a role of SRB in the environments (Kunin *et al.*, 2008).

New development of metagenomics expanded our knowledge into the

evolution, ecology and function of not only hidden bacteria in nature but also novel candidate phyla (Brown *et al.*, 2015, Nobu *et al.*, 2015, Sekiguchi *et al.*, 2015), that are sequenced with no cultivated representatives. Although candidate phyla are present in low abundance (Elshahed *et al.*, 2008), they may have important ecological roles in several different environments (Knittel & Boetius, 2009, Yamada *et al.*, 2011, Farag *et al.*, 2014, Gies *et al.*, 2014). Metagenome have yielded partial or nearly-complete genome for several candidate bacterial phyla, and recovered genomes provided information to construct metabolic models that explain the physiology of microbes (Glöckner *et al.*, 2010, Albertsen *et al.*, 2013, McLean *et al.*, 2013, Kirkegaard *et al.*, 2016, Nobu *et al.*, 2016).

The aim of this chapter was to investigate diversity and potential function of sulfate-reducing bacteria using metagenome data to test the hypothesis that many undiscovered bacteria may exist and play important roles with respect to unique environmental conditions. The study was focused on two questions: what kinds of metabolism and sulfate reduction-related metabolic potential exist in the bottom of FRX? Who is the key player for sulfate reduction in the bottom of FRX?

To address the questions, nearly-complete genomes related with sulfate reducing bacteria were extracted from the bottom depth (15m) of Lake Fryxell, which showed rare bacterial phyla and sulfate reduction.

3.2. Materials and methods

3.2.1. Sample collection and DNA extraction

Lake Fryxell (FRX) is one of the representative ice-covered lakes in Taylor Valley of Dry Valleys, East Antarctica (Figure 3.1). Lake water sample was collected from 15 m of FRX in November, 2012. To differentiate the particle-associated (>3.0 μm;3MA) and free-living bacterial assemblages (0.2 to 3.0 μm;3MB) like as community analysis in Chapter 2., water sample was sequentially filtered through 3.0 μm (ADVANTEC, Japan) and 0.2 μm (Millipore, Germany) membrane filters at the site of FRX. The filters were stored at -20°C during shipment from Antarctica to South Korea, then they were stored at -80°C until analysis. Genomic DNA was extracted from half of each filter membrane, capturing size of > 3.0 μm (3MA) and 3.0-0.2 μm (3MB) bacteria, about 500 ml portion using FastDNA® SPIN kit (MP Biomedicals, Illkirch, France) following the standard protocol.

3.2.2. Metagenome shotgun sequencing, read trimming, *de novo* assembly and data binning

DNA libraries were prepared for extracted DNA from two filters (3MA and 3MB) of FRX 15 m using Truseq Nano DNA LT Kit (Illumina) according to the manufacturer's protocol, and paired-end sequenced using the Illumina Miseq platform (2 × 300 bp). Low quality sequences were trimmed out using Skewer v0.2.2 (Jiang *et al.*, 2014) based on options –m pe –Q 30 –q 25 –l 200

(Figure 3.2). Metagenome assembly was carried out with Ray-meta with kmer size 51 (Boisvert *et al.*, 2012), and contig binning was processed by CONCOCT software (Alneberg *et al.*, 2013) after read length below 5 kb were removed. Conserved marker proteins (open reading frames; ORF) were identified with Prodigal software with metagenome option (Hyatt *et al.*, 2010), and validation of population genome assemblies has been done using essential single-copy genes with Clustering Orthologous Genes (COG) databases. For draft genome analysis, metagenome clusters were extracted from each of 3MA and 3MB by three fiducial categories, being sulfate reduction-related taxa, containment of all 36 essential single-copy genes and 16S rRNA gene.

3.2.3. Community profiling with 16S rRNA gene and genome analysis

The 16S rRNA gene sequences were clustered into operational taxonomic unit (OTU) based on 97% cutoff, and taxonomic classification was conducted using the Naïve Bayesian Classifier in mothur against the EzTaxon-e database (Kim *et al.*, 2012). All 16S rRNA gene sequences of archaea, chloroplast and mitochondria were removed.

To identify metabolic pathways, amino acid sequences of extracted genome were submitted to KEGG Automatic Annotation Server (KAAS) (Moriya *et al.*, 2007) by list of the domain Bacteria, and were run in bidirectional mode of draft genome. Metabolic information between ORF calling and KAAS were compared and summarized. Also, module completion

ratio calculated and compared between two filters with Metabolic And Physiological potential Evaluator (MAPLE-2.3.0) (Takami *et al.*, 2016).

3.2.4. Phylogenetic tree construction

To construct a phylogenetic tree, the partial 16S rRNA gene sequences were extracted from draft genomes, and the sequences were compared to publicly available representative SRB. The quality of alignment was checked with MUSCLE algorithm (v3.8.31) (Edgar, 2004), with manually editing using Phydit. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model (Nei & Kumar, 2000). The tree with the highest log likelihood (-15554.0140) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 68 nucleotide sequences. There were a total of 1045 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013). Bootstrap value was tested in 1000 replications (Felsenstein, 1985) and the outgroup of the tree for 16S rRNA gene was used 16S rRNA gene of sulfate reducing archaea.

3.3. Results

3.3.1. Bacterial community profiling using 16S rRNA gene

A total of available read pairs is 8,363,642 for 3MA and 20,051,231 for 3MB samples with Q30 of quality trimming, which recovered 67.44% and 83.43% from raw read pairs, respectively. In general, diverse bacterial phyla were present in both of size fraction samples, 3MA and 3MB (Figure 3.3), but the distribution of major bacterial phyla were differed by samples. In 3MA, candidate division WM88 (WM88) was predominant with 50% of relative abundance, followed by *Deltaproteobacteria* at 7%, *Alpaproteobacteria* at 5.5%, and candidate division JS1 (JS1) at 5%. Also, about 19 phyla were present in low abundance. Unlikely, in 3MB, JS1 was dominant as 20%. The other abundant bacterial phyla were *Deltaproteobacteria* at 15%, WM88 at 8%, and SAR 406 at 6%. Also, other and unclassified Bacteria accounted for higher portion than 3MA.

3.3.2. Comparison of metabolic pathway module between two size-fraction samples

Representative central carbohydrate metabolisms were completely contained in 3MA and 3MB such as Embden-Meyerhof Parnas (EMP), glucogenesis, pyruvate oxidation, citrate cycle, and non-oxidative pentose phosphate pathway (PPP) (Figure 3.4). Also, other pathways such as Entner-Doudoroff (ED) and

Wood-Ljungdahl (WL) pathway were constructed in both filter samples. Dissimilatory sulfate reduction module was calculated to be 100% for two samples.

Also, the ratio of most modules for nucleotide and amino acid was similar between 3MA and 3MB samples, including purine metabolism, branched-chain amino acid, cofactor and vitamin biosynthesis and so on. But, number of the F420 biosynthesis module in 3MB was much higher than in 3MA. Certain modules were found in specific samples. While nitrogen fixation module and thiosulfate oxidation module existed in only 3MA sample, assimilatory nitrate reduction and secondary metabolism such as cumate degradation and flavanone biosynthesis, were only found in 3MB sample. However, these module information did not explain which bacteria in the sample related with defined module because the data was derived from metagenomes in FRX.

3.3.3. Draft genomes and metabolic characterization for sulfate reducing bacteria

A total of 85 draft genome clusters was recovered from two size fraction samples in 15 m of FRX; 19 genome clusters (MGC_0 to MGC_18) from 3MA and 66 genome clusters (MGC_0 to MGC_65) from 3MB based on MetaGenome Cluster (MGC). Above all, to curate nearly complete genomes related to sulfate reduction, eight MGCs were extracted from each of 3MA and

3MB based on the presence of all 36 essential single-copy genes with at least one copy of the gene (Table 3.1). After adding two more conditional sorting of 8 genome clusters as being sulfate reduction-related taxa. Taking 16S rRNA gene into a consideration, a total of four nearly complete genomes were obtained, two (MGC_0 and MGC_14) from 3MA and two (MGC_9 and MGC_61) from 3MB (Table 3.2). However, there was an exception for MGC_0 in 3MA, which satisfied two categories, being sulfate reducer with single-copied genes, however, it carried no 16S rRNA gene. It was the only cluster that was classified to order *Deltaproteobacterium* only, whereas other taxa were assigned to several clusters.

These four clusters were identified to belong to the order *Deltaproteobacterium* (belonging to the class *Deltaproteobacteria*) for MGC_0, order WM88_o (belonging to candidate division WM88) for MGC_14, and order *Desulfobacterales* (belonging to *Deltaproteobacteria*) for MGC_61.

Three of four clusters were each assigned to different bacterial orders: the cluster MGC_0 was identified to belong to the order *Deltaproteobacterium* (belonging to the class *Deltaproteobacteria*), the cluster MGC_14 was showed to belong to the order WM88 (belonging to the candidate division, WM88), and the cluster MGC_61 was assigned to the order *Desulfobactrales* belong to the class *Deltaproteobacteria*.

But, MGC_9 genome cluster was assigned to two different populations of SAR 406 and order *Syntrophobacterales* (belonging to the order

Deltaproteobacteria) which was further analyzed by phylogenetic analysis. The proportion of taxa for four genomes were shown in Figure 3.5. The order *Deltaproteobacterium* was accounted for 2% and order WM88 was accounted for 51% and in 3MA sample, extracting MGC_0 and MGC_14, respectively. In 3MB, extracting MGC_9 and MGC_61, order *Syntrophobacterales* and *Desulfobacterales* constituted for 3% and 4% in relative abundance, respectively.

Amino acids of four genomes were analyzed by KAAS based on BLAST searches, and central metabolic pathways were assessed. All genomes encoded Embden-Meyerhof-Parnas (EMP) as glycolysis pathway with the non-oxidative of the pentose phosphate pathway (PPP) (Figure 3.6, Figure 3.7, and Table 3.2 for gene annotation in detail). Also, all genomes contained propionyl degradation and acetyl CoA fermentation pathways. The genomes were involved with not only a variety of hydrogenases, but also oxidative stress proteins. All genomes have proteins encoding for rod-shape determining, type IV pilus and partial flagellar proteins. Cell envelopes were characterized with outer membrane systems.

From three genomes (MGC_0, MGC_9 and MGC_61), two representatives of energy metabolism such as Wood-Ljungdahl (WL) pathway and dissimilatory sulfate reduction were well constructed. And, tricarboxylic acid (TCA) cycle was well defined in three genomes as well. In MGC_14 (WM88), however, many proteins for WL and TCA were not detected, and it

only had Sat enzyme for the first step in sulfate reduction, which converts sulfate to APS. None of four genomes was associated with respiration with nitrate/nitrite, iron, and oxygen. The four genomes encoded different electron-carrying proteins and different abilities for energy store and energy degradation system (Table 3.2).

3.3.4. Phylogenetic positions of four draft genomes

Phylogenetic analysis based on the full-length of 16S rRNA gene sequences, except for MGC_0, indicated that 16S rRNA gene of MGC_61 genome was well affiliated to *Deltaproteobacteria* group and MGC_14 genome was classified to novel WM88 clade (Figure 3.7). The phylotype from two draft genomes was shown to be different clades from previously known relatives as Bacteria. On the other hand, MGC_9 genome was shown to have a distinct clade from both SAR 406 and order *Syntrophobacterales* of *Deltaproteobacteria*, which were the sequence similarity results from taxa assignments based on EzTaxon-e DB. MGC_9 genome was excluded from draft genome analysis of sulfate reducing bacteria because the genome was not well defined as sulfate reducing bacteria, mixed with several population groups.

3.4. Discussion

This is the first metagenome approach to construct the genome of sulfate reduction-related bacteria and to research their potential ecological function in

the bottom of Lake Fryxell in McMurdo Dry Valleys, Antarctica. Many previous studies have been focused on monitoring of microbial community composition (Vick-Majors *et al.*, 2013, Kwon *et al.*, 2017) and physicochemical characteristics (Green & Lyons, 2009) using limited samples and cultivate methods (Karr *et al.*, 2005). This study has reconstructed genomes and investigated their potential ability of sulfate reduction using metagenome shotgun sequencing, which allow us to peer into the genome without cultivation.

Total of four sulfate reduction-related genomes were successfully retrieved by data binning. Two (MGC_0 and MGC_61) genomes were assigned to the order *Deltaproteobacteria*, which was well known as sulfate-reducing bacteria, and another (MGC_14) was classified to candidate division WM88, which is not well defined yet.

Sulfate reduction metabolism

WM88 genome (MGC_14) encoded genes for transporting systems such as Rnf protein complex, sulfate transporters, cytochrome c, and other hydrogenases, which are important for sulfate reduction (Pereira *et al.*, 2011). These complexes provide electrons and hydrogen ions to sulfate reductases. The genome also has genes encoding DsrC and Sat, which transport sulfate and convert sulfate to APS, respectively (Muyzer and Stams, 2008).

However, WM88 genome (MGC_14) did not contain any genes for key pathways of sulfate reduction such as Apr and Dsr, which are responsible

for final steps of sulfate reduction as key enzymes. These findings indicate that WM88 may not be directly involved with sulfate reduction although it dominantly presents in FRX. In previous studies, WM88-like organisms, so called Hyd24-12, the closest taxa to WM88, may relate with sulfur transformations (Kirkegaard *et al.*, 2016) according to their detection in sulfur rich environments such as hydrothermal vents and sediments (Schauer *et al.*, 2011, Pjevac *et al.*, 2014). Moreover, WM88 potentially play a role as a syntrophic bacteria with other SRB, that is, WM88 could be a provider of electrons, hydrogen and formate (Embree *et al.*, 2015).

Other two deltaproteobacterial genomes (MGC_0 and MGC_61) were well defined as SRB by previous studies. The order *Deltaproteobacterium* (MGC_0) is capable of sulfate-dependent propane and butane metabolism under anaerobic condition (Kniemeyer *et al.*, 2007). The genome, MGC_61, was identified as sulfate reducer for it belong to the order *Desulfobacterales* that includes known hydrogenotrphs (Burow *et al.*, 2012), consuming H_2 microorganisms. These two genomes also had all protein complexes for transporting electrons and hydrogen ions as carried by WM88 strains. Moreover, two genomes belonging to *Deltaproteobacteria* have key genes for the necessary pathways of sulfate reduction, including Sat, Apr and Dsr, producing sulfide as a final product.

In addition, two deltaproteobacterial genomes encode genes of DsrMKJOP complex, which is a transmembrane complex with redox subunits

in the periplasm (Pester *et al.*, 2012). Specially, DsrMK is essential for sulfite reduction, and it may be related with menaquinol oxidation and reduction of a cytoplasmic substrate (Oliveira *et al.*, 2008). These findings support that the order *Deltaproteobacteria* may play a key role for sulfate reduction in FRX (Kirkegaard *et al.*, 2016). Also, whole pathways of sulfate reduction may be completed by the interaction between WM88 and *Deltaproteobacteria*.

Central carbohydrate metabolism

The genome, MGC_14, belonging to the WM88 group contained nearly complete EMP with non-oxidative PPP, only missing two enzymes each for EMP and PPP. It means that MGC_14 genome may potentially catabolize a wide range of monosaccharides such as pentoses and hexoses to pyruvate (Stincone *et al.*, 2015). Many genes related to TCA cycle were not detected in the genome, but had a pyruvate ferredoxin oxidoreductase. These results indicate that pyruvate may be converted to acetyl-CoA instead, in absence of TCA (Menon and Ragsdale, 1997).

Deltaproteobacterial genomes (MGC_0 and MGC_61) had a complete EMP combined with the non-oxidative PPP. MGC_0 and MGC_61 genomes encoded genes for a complete TCA cycle. This indicates that two genomes also potentially catabolize a wide range of sugars like WM88 does, supplying the energy of ATP and NADH form (Stincone *et al.*, 2015). In addition, the genomes could convert not only pyruvate into acetyl-CoA by a

pyruvate:ferredoxin oxidoreductase, providing additional reduced ferredoxin (Menon & Ragsdale, 1997), but also acetyl-CoA into acetate by two enzymes such as phosphate acetyltransferase and acetate kinase, providing additional ATP (Latimer & Ferry, 1993).

Other energy metabolism

There is lack of information for WL pathway in WM88 genome (MGC_14), but the genome is potentially related to degrading propionate through the methylmalonyl-CoA (Mmc) pathway, encoding Mmc mutase, epimerase and decarboxylase gene, which produce NADH (Nobu *et al.*, 2016). In addition, this genome contains all genes for glycogen biosynthesis and catabolism pathway, which are the main strategy for metabolic storage (ie. carbon and energy storage) (Wilson *et al.*, 2010). MGC_14 genome might accumulate the polyglucan under conditions of excess of carbon source and deficient of other nutrients (Eydallin *et al.*, 2007).

For two genomes related to deltaproteobacterial group, they may conserve the energy and synthesis of acetyl-CoA, encoding pathways for fixation of CO and CO_2 via the reductive acetyl-CoA or the WL pathway (Gies *et al.*, 2014). WL pathway is used for acetate catabolism by SRB (Schauder *et al.*, 1988). They also can use propionate and store energy using Mmc and glycogen biosynthesis pathways like WM88 genome.

Morphology and mobility

Suggestion of rod shape morphology of all three genomes was supported by Mre operons within the genome (Table 3.3), encoding proteins for the formation of membrane-bound actin filaments (Kruse *et al.*, 2005). Three genomes had an ability of adherence associated with not only flagella-related proteins but also type IV fimbriae or pili genes. These genes support the attachment to the surface of the substrate or other bacteria (Giltner *et al.*, 2012). Also, these fimbriae/pili allow to move by gliding along the substrate and to form the biofilm by adsorption with the extracellular polymeric substances (Jin *et al.*, 2011).

Moreover, for two deltaproteobacterial genomes, there were genes encoding flagella-related proteins, suggesting the mobility of the bacteria that carry the genome. Flagella protein, FliJ acts as a general chaperone to control export of substrates in the cytoplasm (Minamino *et al.*, 2000). FlgD is related to basal-body modification (Ohnishi *et al.*, 1994), and FliK functions as the checkpoint for the optimal length of flagella hook substructure (Waters *et al.*, 2007). The characteristics of cell envelope were determined with an archetypical diderm with lipopolysaccharides (Albertsen *et al.*, 2013). None of three genomes had genes related with spore formation, which was typical feature for non-sporulating and negative bacteria.

Oxidative stress protection and dormancy control

Three genomes, MGC_14, MGC_0 and MGC_61, had gene clusters encoding for a superoxide reductase, catalase, rubrerythrin, and rubredoxin. These genes are concerned with resistance against oxidative stress, and may allow the bacteria to endure in the presence of oxygen (Kantor *et al.*, 2013, Kirkegaard *et al.*, 2016). The three genomes had genes associated with cell dormancy (Hip protein), which affect cell division and interact with cell division genes such as DNA or peptidoglycan synthesis under the lethal condition (Black *et al.*, 1994).

Electron transporting

There are several electron-carrying proteins in four genomes such as iron-sulfur cluster, ferredoxin reductase, cytochrome b, cytochrome c, and flavodoxins. These findings indicate that these genomes are predicted to be able to use diverse electron uptake and transport systems. Energy-conserving hydrogenases make the cells available to constitute a proton motive force (PMF), with the presence of a membrane-embedded hydrogenase, reducing hydrogen ion to hydrogen (Strittmatter *et al.*, 2009) and conserving formate (Wang *et al.*, 2013) by NiFe-hydrogenase. Moreover, a membrane-bound pyrophosphatase is used to generate PMF as well (Kantor *et al.*, 2013). Also, genomes could generate the energy by electron and proton transport with heterodisulfide reductase gene clusters (Pierce *et al.*, 2008).

This was the first study that applied metagenomics to attain genomes and to reconstruct metabolic pathway for sulfate reduction-related genomes in the bottom of Lake Fryxell. Further research is needed to confirm the ability of the genomes in detail such as proteomics. Nevertheless, the findings from this study offer the possible morphological and physiological information even for uncultivated bacteria. Also, these results provide the fundamental information for genomes in the Antarctic lake ecosystem.

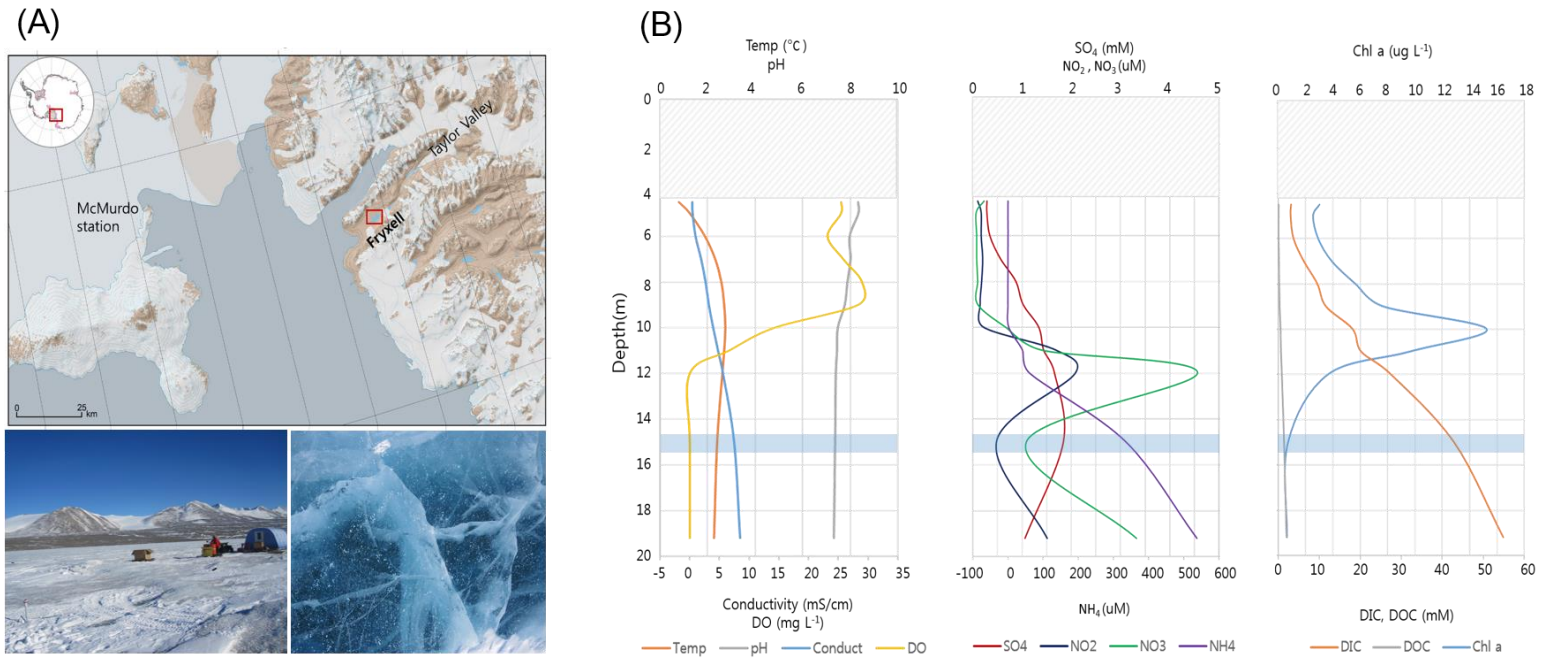

Figure 3.1. Site map for Lake Fryxell in Taylor Valley, Antarctica. (A) site description (B) and biogeochemical depth profiling.

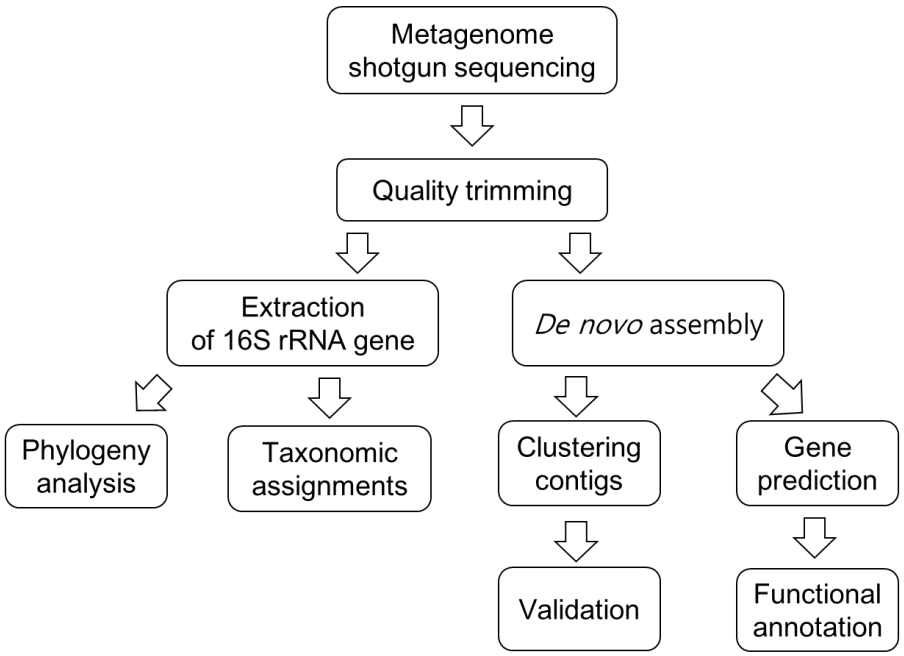

Figure 3.2. Overview of the pipeline to obtain high-quality population genomes from metagenome shotgun data.

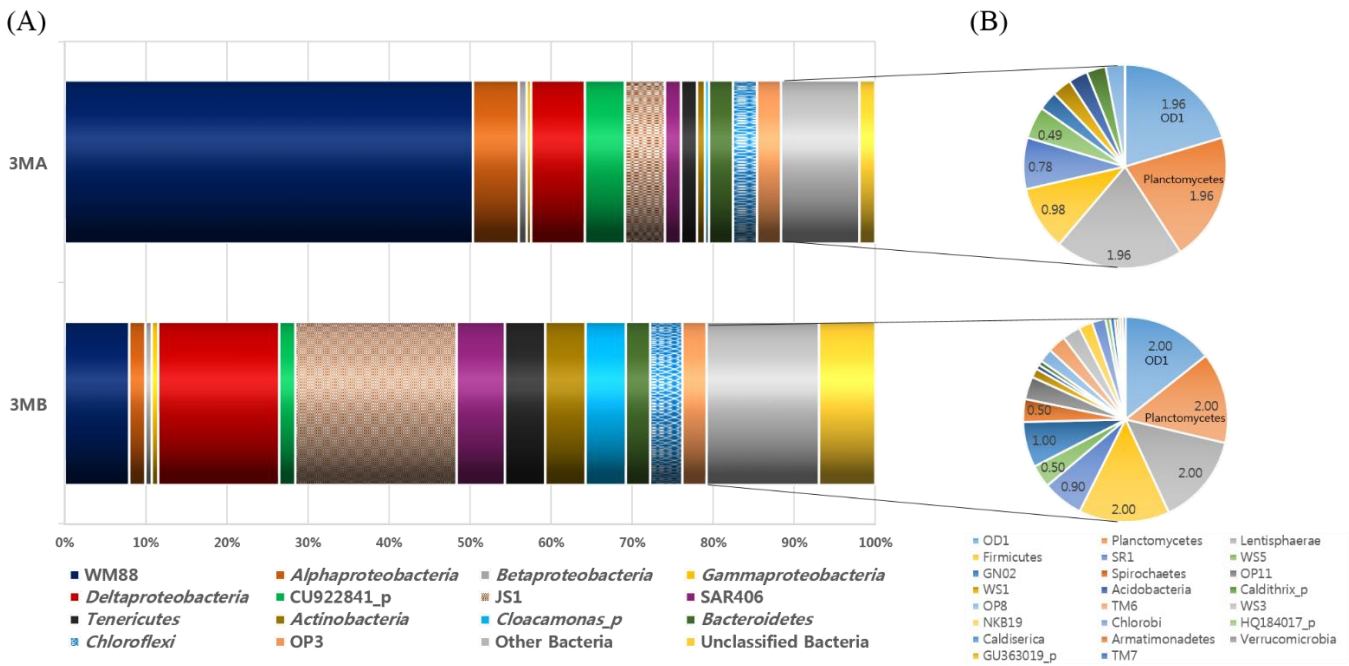

Figure 3.3. Bacterial community profiling based on 16S rRNA gene in metagenome shotgun data from 15m of Lake Fryxell. 3MA and 3MB denote the > 3.0 µm and the 0.2 to 3.0 µm size fraction, respectively. Other bacteria denote the group of relatively rare phyla with a relative abundance of ≤ 2% in each sample. (A) community structure in phylum level (B) phyla list belonging to other bacteria.

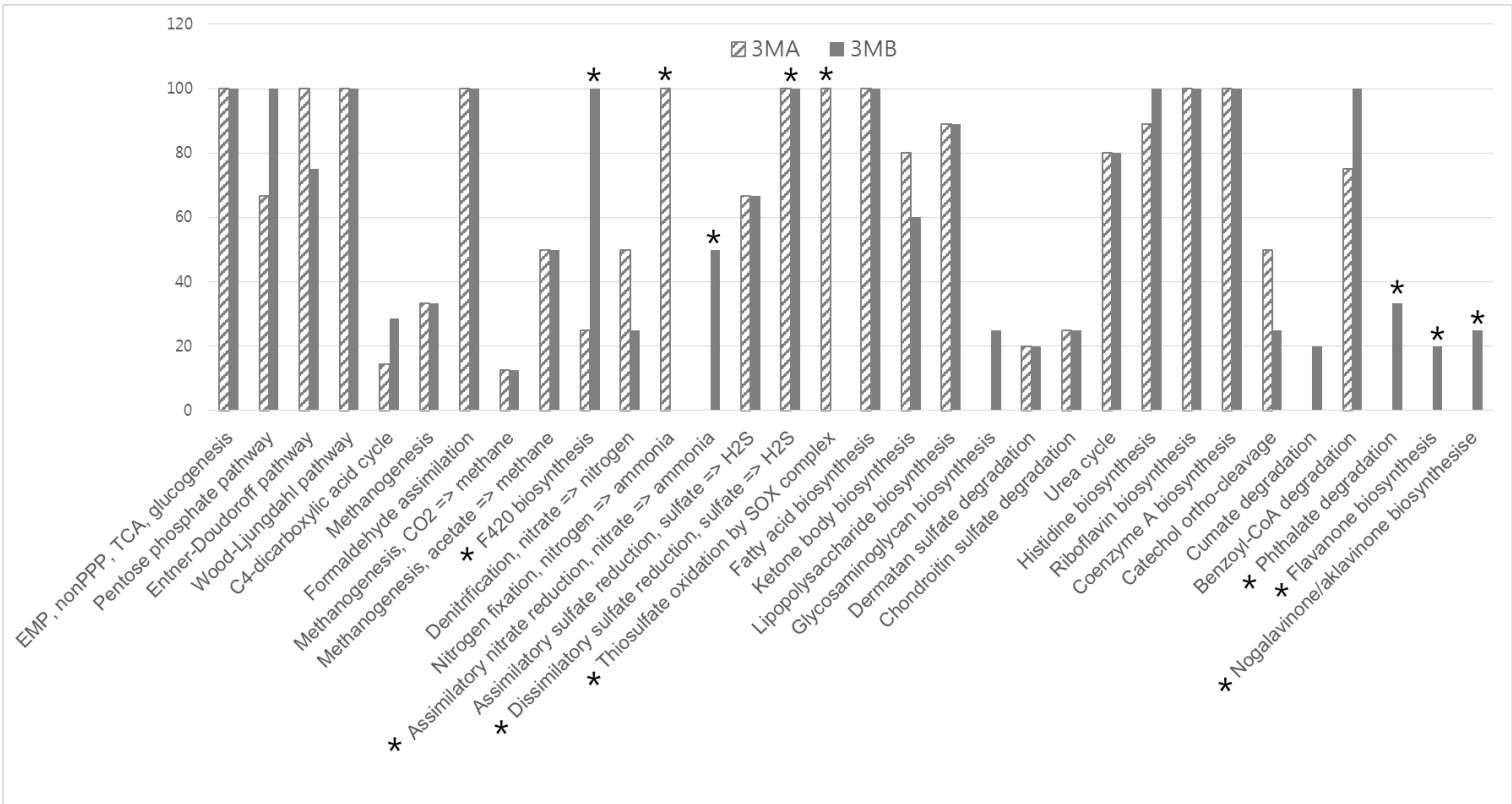

Figure 3.4. Comparison of metabolic pathway module between 3MA and 3MB.

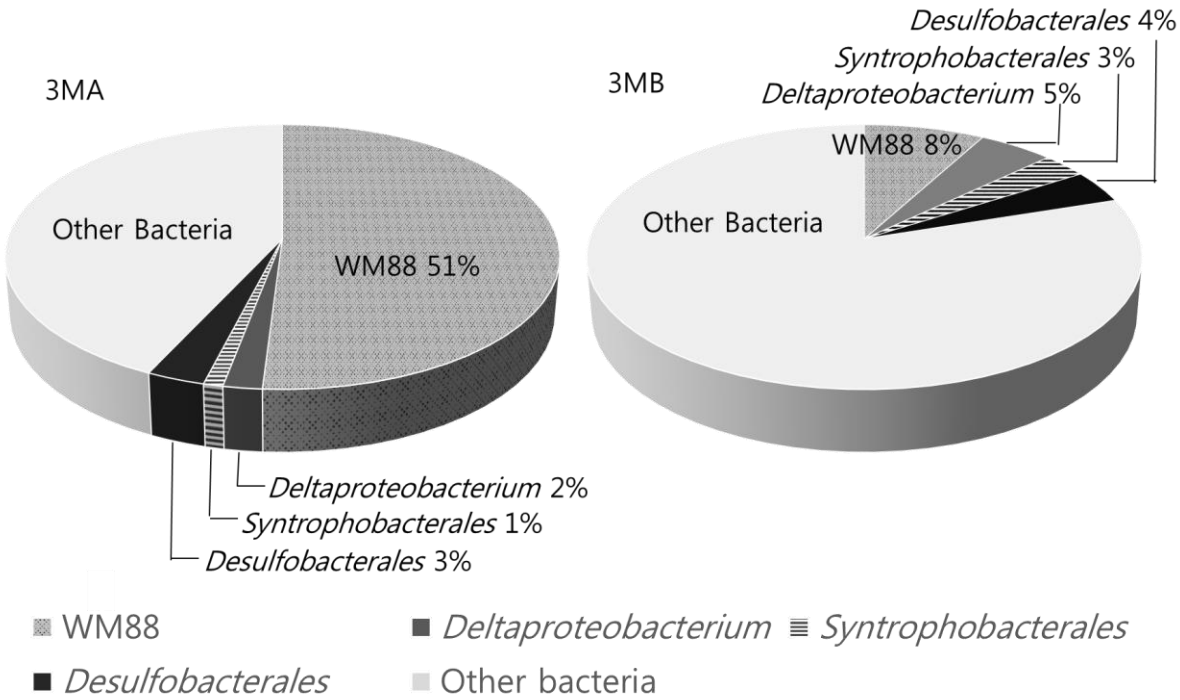

Figure 3.5. The proportion of taxa related with four draft genomes in community profiling between 3MA and 3MB.

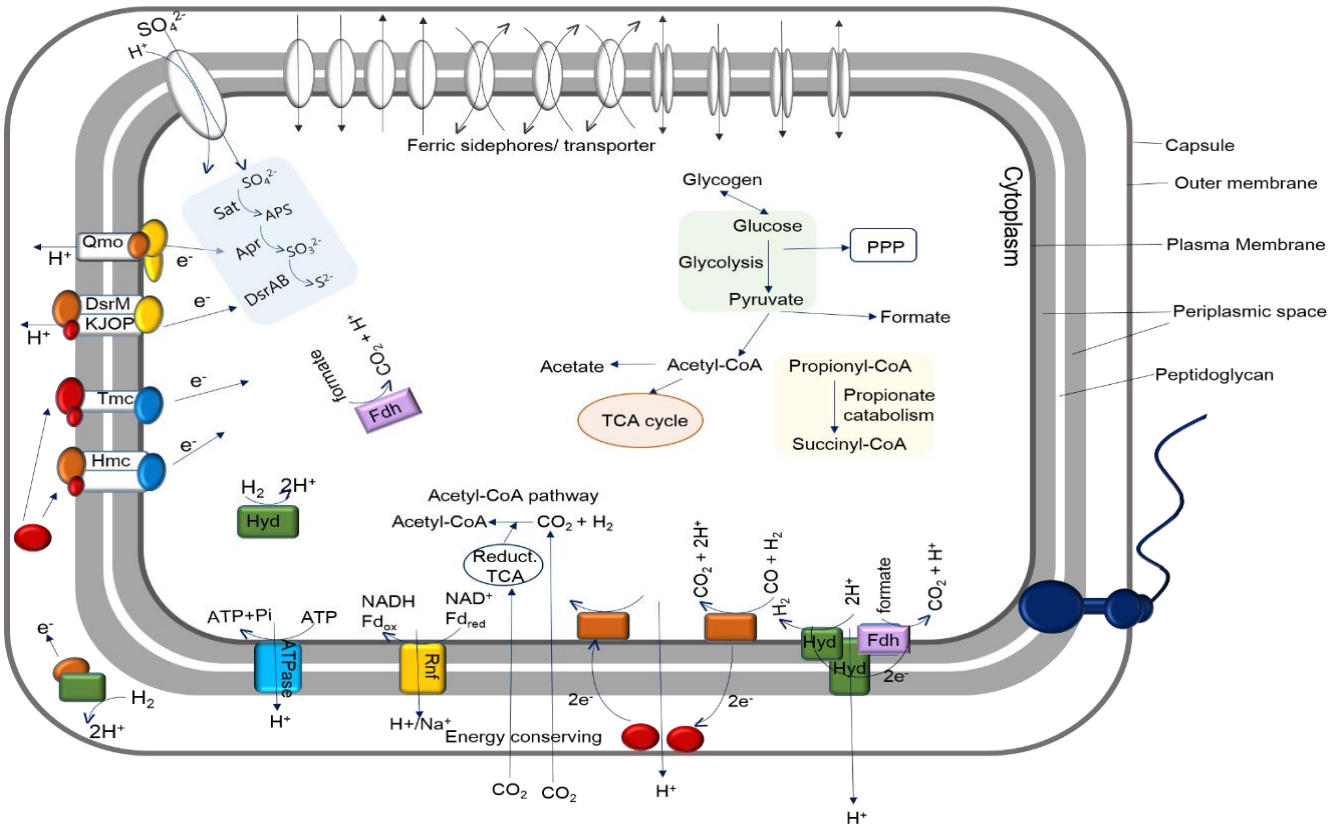

Figure 3.6. Metabolic model for two deltaproteobacterial draft genomes recovered from data binning.

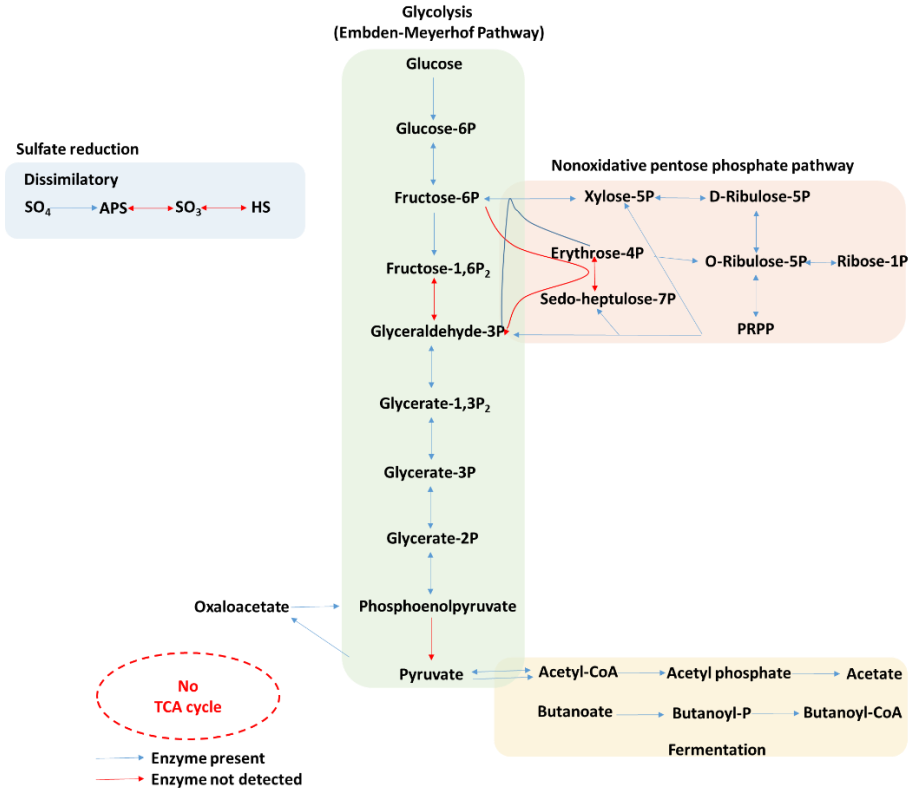

Figure 3.7. Cell diagrams depicting central carbon metabolism, proteins, and transporters in three draft genomes of SRB (1/3). MGC_14 genome was retrieved from 3MA, which was assigned to candidate phylum WM88 based on 16S rRNA gene sequences. Red arrow denotes that the enzyme was not detected in this genome.

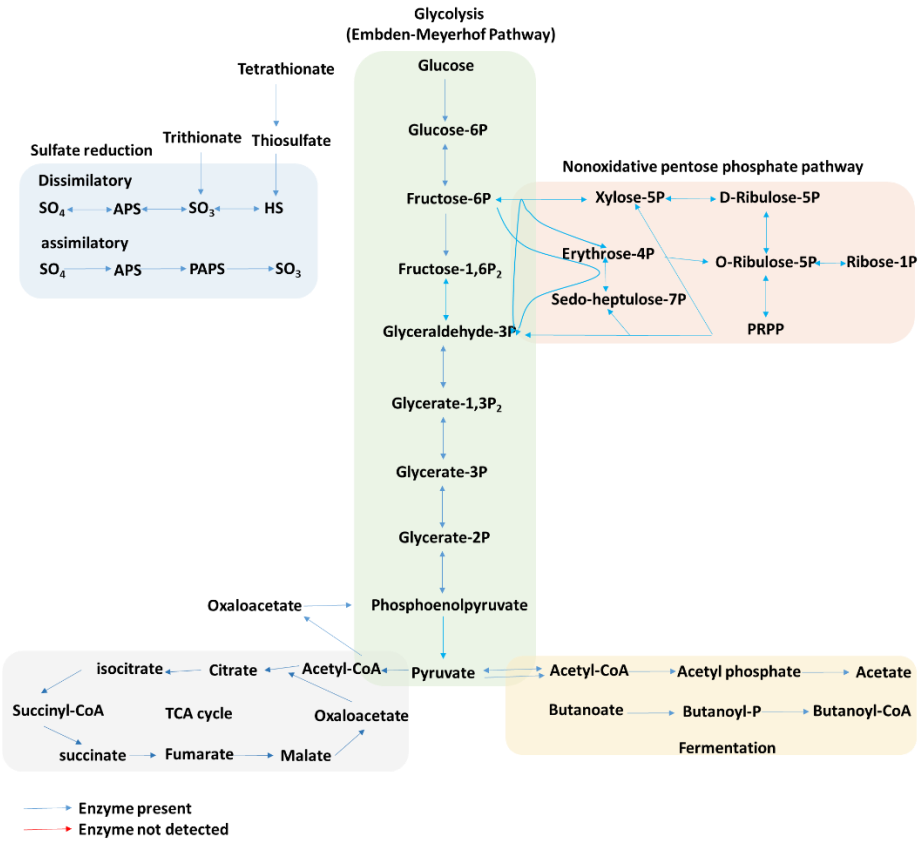

Figure 3.7. Cell diagrams depicting central carbon metabolism, proteins, and transporters in three draft genomes of SRB (2/3). MGC_0 genome was retrieved from 3MA, which was assigned to the order of uncultured deltaproteobacterium belonging to the class *Deltaproteobacteria* based on 16S rRNA gene sequences. Red arrow denotes that the enzyme was not detected in this genome.

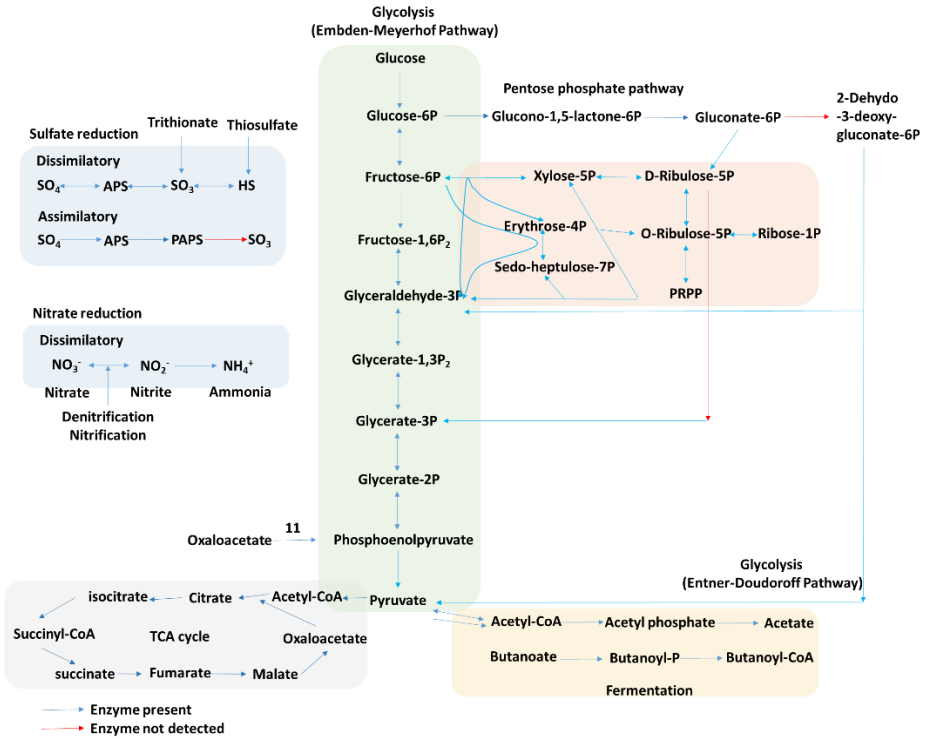

Figure 3.7. Cell diagrams depicting central carbon metabolism, proteins, and transporters in three draft genomes of SRB (3/3). MGC_61 genome was retrieved from 3MB, which was assigned to the order *Desulfobacterales* of the class *Deltaproteobacteria* based on 16S rRNA gene sequences. Red arrow denotes that the enzyme was not detected in this genome.

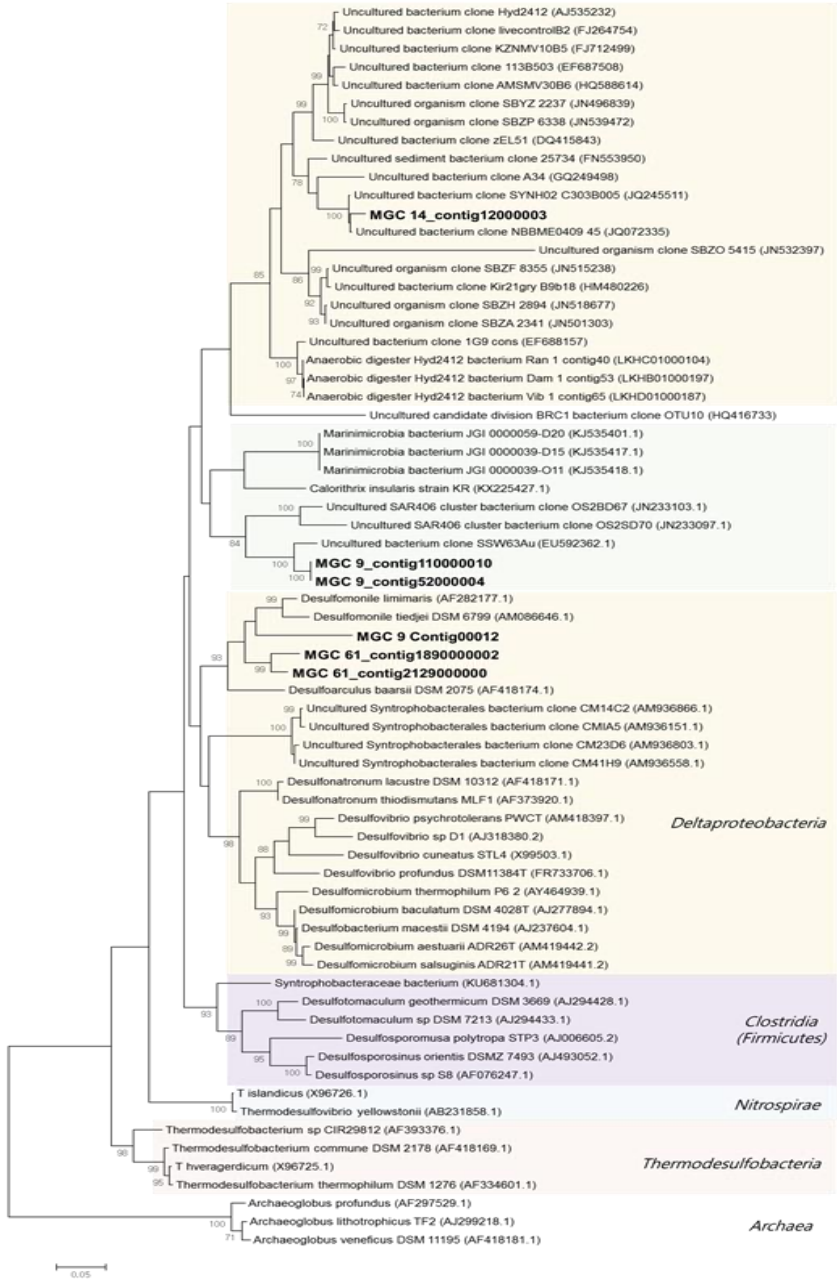

Figure 3.8. Phylogeny of sequences affiliated with three MGC genomes, MGC_9, MGC_61 and MGC_14. The tree was calculated with 16S rRNA gene using Maximum Likelihood algorithm and 1,000 bootstrap values.

Table 3.1. Genome information for 16 genome clusters from this study

Sample	Cluster	No. of contigs	Total size (bp)	Copy no. of 16S rRNA genes	RPKM of each genome cluster	Avg. depth	Ratio in meta genome data	Closest taxa based on 16S rRNA gene	Closest taxa based on single copy COG gene	Copy no. of single copy gene	Sulfate reduction
3MA	MGC_0	366	6,481,577	0	9.426	23.09	3.27	-	Deltaproteobacteria(uncultured delta proteobacterium)	≥1	o
	MGC_1	252	3,156,231	1	7.802	19.55	1.32	Mollicutes (Acholeplasma)	Mollicutes (Acholeplasma)	≥1	x
	MGC_4	164	3,534,320	0	12.335	24.21	2.33	-	Detaproteobacteria(syntrophobacterales)	≥1	o
	MGC_6	101	1,746,895	0	9.728	24.54	0.91	-	Mollicutes (Haloplasmatales)	≥1	x
	MGC_8	82	2,320,144	1	10.399	25.74	1.29	SAR406 (Marinimicrobia)	SAR406 (Marinimicrobia)	1	x
	MGC_12	101	2,800,835	0	8.438	19.09	1.27	-	WWE1(cloacamonas)	≥1	x
	MGC_14	49	2,677,987	1	159.397	392.43	22.85	WM88	WM88	1	o
	MGC_18	259	3,040,170	0	8.196	21.10	1.33	-	WWE1(cloacamonas)	1	x
3MB	MGC_9	105	5,273,661	3	19.32	154.25	6.61	Sar, Deltaproteobacteria(syntrophobacterales)	SAR406, Deltaproteobacteria(syntrophobacterales)	≥2	o
	MGC_12	53	2,738,228	1	8051	62.39	1.51	WM88	WM88	1	o
	MGC_23	163	6,611,351	1	11.84	71.98	5.07	WWE1(cloacamonas)	WWE1(cloacamonas)	2	x
	MGC_25	151	3,857,861	0	3.04	22.37	0.76	-	SAR406 (Marinimicrobia)	1	x
	MGC_50	101	2,030,790	1	6.91	53.34	0.91	Mollicutes	Mollicutes (Acholeplasma)	≥1	x
	MGC_59	251	6,821,438	3	6.34	45.72	2.81	SAR406, Mollicutes	SAR406, Mollicutes	≥2	x
	MGC_61	721	13,361,024	2	5.19	37.58	4.49	Desulfobacterium	Deltaproteobacteria(Desulfobacterales)	>2	o
	MGC_62	443	8,220,222	0	3.59	26.73	1.91	Desulfobacterium	Deltaproteobacteria(Desulfobacterales)	≥1	o

Table 3.2. Genome information for three draft genomes from this study (1/4)

	Gene name	MGC_0	MGC_14	MGC_61	EC number
Embden Meyerhoff Parnas	Glucose-6-phosphate isomerase	+	+	+	5.3.1.9
	phosphoglucose/phosphomannose isomerase	-	-	-	
	Mannose-6-phosphate isomerase	+	+	+	5.3.1.8
	6-phosphofructokinase	+	+	+	2.7.1.11
	Predicted Fructose-bisphosphate aldolase	+	+	+	4.1.2.13
	Triosephosphate isomerase	+	-	+	5.3.1.1
	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase	+	+	+	1.2.1.12
	Phosphoglycerate kinase	+	+	+	2.7.2.3
	Glyceraldehyde-3-phosphate: ferredoxin oxidoreductase	-	-	+	1.2.7.6/1.2.1.9
	phosphoglycerate mutase	+	+	-	5.4.2.1
	Enolase	+	+	+	4.2.1.11
	Pyruvate kinase	+	-	+	2.7.1.40
	pyruvate phosphate dikinase/PEP synthetase	+	+	+	2.7.9.1/2.7.9.2
Pentose phosphate	Glucose-6-phosphate 1-dehydrogenase	-	-	-	1.1.1.49
	6-phosphogluconolactonase	-	-	+	3.1.1.31
	6-phosphogluconate dehydrogenase	-	-	-	1.1.1.44
	Ribose 5-phosphate isomerase	+	+	+	5.3.1.6
	Ribulose-phosphate 3-epimerase	+	+	+	5.1.3.1
	Transketolase	+	+	+	2.2.1.1
	Transaldolase	+	-	+	2.2.1.2
	phosphoketolase	-	-	-	4.1.2.9
Pyruvate metabolism	Pyruvate formate-lyase	+	-	+	2.3.1.54
	Pyruvate:ferredoxin oxidoreductase	+	+	+	1.2.7.1
	Glucose-1-phosphate adenylyltransferase	+	+	+	2.7.7.27
	Glycogen synthase	+	+	+	2.4.1.21
Starch and sucrose metabolism pathway	1,4-alpha-glucan branching enzyme	+	+	+	2.4.1.18
	Glycogen phosphorylase	+	+	+	2.4.1.1
Propionyl-CoA_to_Succinyl-CoA_Module	Methylmalonyl-CoA mutase (Mmc)	+	+	+	5.4.99.2
	Methylmalonyl-CoA epimerase	+	+	+	5.1.99.1

Table 3.2. Genome information for three draft genomes from this study (2/4)

	Gene name	MGC_0	MGC_14	MGC_61	EC number
Fermentation	Phosphate acetyltransferase/ phosphotransacetylase	-	-	+	2.3.1.8/ 2.3.1.222
	Phosphate acetyltransferase	-	+	+	2.3.1.19
	Acetate kinase	-	+	+	2.7.2.1
	Acetyl-CoA synthetase (ADP-forming)	+	+	+	6.2.1.13
	malate dehydrogenase	+	-	+	1.1.1.38/37
	Butyrate kinase	-	+	-	2.7.2.7
	Energy-conserving hydrogenase (ferredoxin)	-	+	-	
	Coenzyme F420-reducing hydrogenase	+	+	+	
	NAD-reducing hydrogenase	+	+	+	1.12.1.2
	NADH-ubiquinone oxidoreductase	+	+	+	1.6.5.3
	NADH dehydrogenase	+	+	+	1.6.99.3
	NADH-dependent butanol dehydrogenase A	-	-	-	1.1.1.-
	NAD-dependent formate dehydrogenase	+	+	-	
	Pyruvate formate-lyase activating enzyme	+	-	+	1.97.1.4
	Succinate dehydrogenase	+	-	+	
	Xylulose-5-phosphate phosphoketolase	-	-	-	4.1.2.9
	Alcohol dehydrogenase	+	-	+	1.1.1.1
	L-lactate dehydrogenase	+	-	+	
	D-lactate dehydrogenase	-	-	-	1.1.1.28
	phosphotransbutyrylase	-	+	-	2.3.1.19
Acetyl-CoA_ fermentation_ to_Butyrate	3-hydroxyacyl-CoA dehydrogenase	+	+	+	1.1.1.35
	3-hydroxybutyryl-CoA dehydrogenase	+	+	+	1.1.1.157
	Acetyl-CoA acetyltransferase	+	-	+	2.3.1.9
	Electron transfer flavoprotein	+	+	+	
	Enoyl-CoA hydratase	+	-	+	4.2.1.17
	AMP/thymidine phosphorylase	-	-	-	2.4.2.4/2.4.2.7
AMP salvage	Ribose 1,5 P isomerase	-	+	+	5.3.1.6
	Rubisco	-	-	-	4.1.1.39

Table 3.2. Genome information for three draft genomes from this study (3/4)

	Gene name	MGC_0	MGC_14	MGC_61	EC number
Cell envelope	Outer membrnae system (Yae, Lol)	+	+	+	
Morphology	Rod shape-determning protein (Mre, Rod)	+	+	+	
	Type IV pilus	+	+	+	
	Flagellar protein	(K,J,Q,S)	(FliK,FlgD, FliJ)	(FliK,FleS,FleN ,Fmot,FleQ, FliJ,FlgK,FleN)	
Sulfate reduction	Adenylylsulfate reductase	+	-	-	1.8.99.2
	Dissimilatory sulfite reductase	+	-	+	1.8.99.1
	Sulfate adenylyltransferase (ATP sulfurylase)	+	+	+	2.7.7.4
	Adenylylsulfate kinase	+	+	+	2.7.1.25
Hydrogenase	Butyryl-CoA dehydrogenase	+	+	+	1.3.99.2
	Formate dehydrogenase	+	+	+	1.2.1.2
	Fe-S-cluster-containing hydrogenase	+	-	+	
	[Ni/Fe] hydrogenase	+	+	+	
	Coenzyme F420-reducing hydrogenase	+	+	+	
	NADP-reducing hydrogenase	+	-	+	
	Nickel-dependent hydrogenase	+	+	+	
	Periplasmic [Fe] hydrogenase	+	+	+	1.12.7.2
	Ni-dependent hydrogenase	-	-	+	1.12.99.6
Electron transport	Ferredoxin:NAD oxidoreductase	-	-	-	1.18.1.3
	Flavodoxin reductases (ferredoxin-NADPH reductases)	+	-	+	
	Heterodisulfide reductase	+	+	+	
	Pyruvate-flavodoxin oxidoreductas	+	+	-	
	Ferredoxin--NADP(+) reductase	-	-	-	1.18.1.2
	NADH dehydrogenase (+)	+	-	+	1.6.99.3
	cytochrome b6	+	-	+	
	cytochrome c	+	+	+	
	Arsenate reductase	+	+	+	1.20.4.1
	CoB--CoM heterodisulfide reductase	+	+	+	
	NADH-ubiquinone oxidoreductase chain B	+	+	+	1.6.5.3
	DsrMKJOP complex	+	-	+	
	Rnf protein complex	+	+	+	

Table 3.2. Genome information for three draft genomes from this study (4/4)

	Gene name	MGC_0	MGC_14	MGC_61	EC number
Oxidative stress	Superoxide reductase	+	+	+	1.15.1.2
	Rubrerythrin	+	+	+	
	Rubredoxin	+	+	+	
	Catalase	+	+	+	1.11.1.6
Proton motive force	ATP synthase (F1F0-type)	+	-	+	3.6.3.14
	ATP synthase (V-type)	-	+	+	3.6.3.14
	Membrane-bound pyrophosphatase, Pyrophosphate-energized proton pump	+	+	+	3.6.1.1
	Ferredoxin	+	+	+	
ABC transporters					
Mineral and organic ion transporters	Tungstate	+	-	-	
	Sulfate	-	+	-	
	Molybdate	+	-	+	
	Spermidine/Putrescine	-	-	+	
	Osmoprotectant	-	-	+	
	Alkanesulfonate	-	-	+	
Oligosaccharide, polyol, lipid transporter	Phospholipid	+	-	+	
	Sorbitol/Mannitol	-	-	+	
Phosphate and amino acid transporters	Branched-chain amino acid	+	-	+	
	Urea	+	-	+	
	Phosphate	-	+	+	
	Neutral amino acid/Histidine	-	-	+	
Metallic caion, iron-siderophore and vitamin B12	Iron complex	+	-	+	
	Zinc	+	+	+	
	Cobalt	+	-	+	
	Nickel	+	-	+	
ABC-2 and other	Biotin	+	-	+	
	Lipoprotein	-	+	+	
	Lipopolysaccaride	+	+	+	
	Ribose/Autoinducer2/D-xylose	+	-	+	
Monosaccharide	Glycerol	+	-	+	
	sn-Glycerol3-phosphate	-	-	+	
Peptide and nickel transporters	Dipeptide/Heme	-	-	+	
	Microcin C	-	-	+	

CHAPTER 4.

Culture-dependent approach:

Culture strategies for sulfate-reducing bacteria in ice-covered Lake Fryxell

4.1. Introduction

Sulfate recycling by sulfate-reducing bacteria (SRB) is important for mineralization of organic matter in terrestrial and aquatic ecosystems as well as the carbon cycle (Knoblauch *et al.*, 1999). The pathway of dissimilatory sulfate reduction is well known as one of the ancient types as biological energy conservation (Shen *et al.*, 2001). Dissimilatory sulfite reductase (Dsr), which consists of tetramer of alpha and beta subunits, is an essential enzyme in the final steps of sulfate reduction to reduce sulfate to sulfide, and all identified SRB carried this enzyme (Wagner *et al.*, 1998). Hence, the sequences of Dsr enzyme have been used to evaluate the SRB diversity and evolutionary aspects due to its high conservation within bacterial species (Klein *et al.*, 2001, Müller *et al.*, 2015). Recent studies have reported high diversity of SRB and their significant roles even in sulfate-depleted environments and diverse environments (Leloup *et al.*, 2006, Schmalenberger *et al.*, 2007).

Lake Fryxell (FRX) is perennially covered with about 5 m of the ice like many other ice-covered lakes in McMurdo Dry Valleys, Antarctica. FRX exhibits a stratified geochemistry in water column owing to absence of mixing of the water body. Attractively, the bottom of FRX is characterized as brackish, anoxic and reduced environment from high concentrations of sulfide and methane in surface (Roberts *et al.*, 2000). In contrast, the concentration of sulfate peaked at the middle of water body, then decreased by deepening depth. Despite of the low intensity of sulfate, sulfate reduction have been detected with

methanogenesis activity in the bottom of FRX (Smith *et al.*, 1993, Karr *et al.*, 2005).

SRB have been cultivated with artificial medium under anaerobic condition in FRX (Smith *et al.*, 1993, Karr *et al.*, 2005). For example, four major groups of SRB were usually detected from FRX water sample, such as *Desulfovibrio*, *Desulfobulbus*, *Desulfobacterium*, and *Desulfotomaculum*. However, there is still limitation in recovering other potential SRB with the artificial medium due to dissimilarity of the geochemical conditions between artificial medium and the actual environment. Given the limitation, it is difficult to define what kind of other SRB exist and how they adapted to the low sulfate concentration in FRX.

The aim of this study is to enrich SRB that are not yet cultured and to better understand the diversity of SRB in ice-covered FRX with the hypothesis that similar biogeochemical characteristics will recover particular SRB existing FRX, and they have a possibility to reveal adaptation history involved in extreme environments. Specific questions were focused on our study: How could uncultivated SRB be enriched in microcosm? How is enriched-SRB phylogenetically different from known SRB based on 16S rRNA gene and Dsr gene? To test the hypothesis, this study integrated two approaches of anaerobic culturing and profiling of 16S rRNA gene fragments from the microcosm. Also, the genomic contexts from the microcosm were investigated with Dsr gene as a key enzyme in sulfate reduction (SR).

4.2. Materials and Methods

4.2.1. Sample collection and microcosm set up

Lake Fryxell (FRX) is located in the Taylor Valley of McMurdo Dry Valleys (MCM), East Antarctica. FRX have been researched for more than 20 years as McMurdo long term ecological research (MCM LTER) project (http://mcm.lternet.edu/) funded by the National Science Foundation (NSF), USA. Twelve liters of bulk water sample was collected from 15 m depth of FRX in December 2014 using Niskin bottle (Figure 4.1), and the bulk water sample was transported FRX to McMurdo Station (MCM) under -20°C. The frozen sample was shipped from MCM to South Korea, and stored at -20°C until processed. Physicochemical and biological characteristics of 15 m depth of FRX was shown in Table 4.1.

Frozen lake water sample was thawed, and sparged with nitrogen gas with purity of 99.99%, then placed inside an anoxic chamber to establish microcosm. For the enrichment culture of SRB, I used two different medium composition for microcosm (Figure 4.2). First, anaerobic medium (DCB-1) was prepared as described by the previous study (Cole *et al.*, 1994). The composition of culture medium was as follows (per liter): NaCl, 1.0 g; $MgCl_2$ *$6H_2O$, 0.5 g; KH_2PO_4, 0.2 g; NH_4Cl, 0.3 g; KCl, 0.3 g; $CaCl_2$ * $2H_2O$, 0.015 g; trace element solution 1 ml; Se/Wo solution, 1 ml; resazurin, 1 mg. After the medium boiled and cooled to room temperature, reductants (Na_2S * $9H_2O$, 0.048 g; L-cysteine, 0.031 g; $NaHCO_3$, 2.52 g;) were added to it. All gas

headspace was flushed with oxygen-free N_2-CO_2 gas, then the final pH of the medium was adjusted to 7.2 to 7.3. The 95 ml of medium was aliquot into 160-ml serum bottles sealed with butyl rubber stoppers and was autoclaved. Five milliliter of anoxic lake water was inoculated to prepared anaerobic medium bottles. Second, the anoxic lake water was directly used as culture medium. Briefly, 100 ml of anoxic lake water was aliquoted to 160-ml serum bottles. Also, additional serum bottles were set up as negative control based on same conditions of two different media. All gas headspace was flushed with oxygen-free N_2-CO_2 gas, followed by sealing with butyl rubber stoppers.

Substrates were added sterilely to all bottles of microcosm: 10 or 1 mM of lactate/formate/acetate/acetate with H_2 as a carbon substrate, 5 or 40 mM sodium sulfate as an electron acceptor, and filter-sterilized vitamin solution. Microcosms prepared in triplicate were incubated at 4°C and 10°C without shaking under the dark condition.

4.2.2. Analytical methods

To withdraw 0.5 ml of liquid from the microcosm, gas-tight glass syringes with gas-tight Teflon valves, and luer lock adapters were used with ultrapure N_2 as carrier gas. Sulfate (electron acceptor) and sulfide (reduction product) were measured using a HACH DR2010 (Colorado, USA). The concentration of both sulfate and sulfide were measured at 0, 6, 15, 37, 65, 99, 137 and 165 days. Sulfate ion formed insoluble barium sulfate turbidity, and the soluble sulfides

formed methylene blue. The turbidity and the intensity indicated the concentration of sulfate and sulfide, respectively.

4.2.3. Serial transfers

After sufficient enrichment, the cultured microcosms were transferred to fresh medium using disposable syringes and needles for the further research. Two media (DCB-1;anaerobic medium and autoclaved-FRX water) were prepared in 160-ml serum bottles under the anoxic condition as fresh medium: FRX water was filtered through 0.2 μm-pore membrane filter (Millipore, Germany), followed by autoclaving. For 1st transferred cultures, 1mM lactate and formate, and 5mM sulfate were used as electron donor and acceptor, respectively. Each transfer culture was maintained for two months for sufficient SRB with regular measurement for the concentration of sulfate and sulfide.

4.2.4. Genomic DNA extraction

To compare dynamics of microbial population between raw FRX lake water and microcosm of FRX, genomic DNA was extracted from both samples after sufficient enrichment. Briefly, 5 ml were withdrawn from each of the 15m depth of untreated FRX water samples (O1 and O2 as a duplicate) and the enriched samples (B1, B2 and B3 as a triplicate) from 5 mM lactate - 5 mM sulfate microcosm at 10°C after 65 days of incubation, followed by filtration onto 0.2 μm-pore membrane filter (Millipore, Germany). Total genomic DNA was

extracted from the filters using FastDNA® SPIN kit (MP Biomedicals, Illkirch, France) following the manufacturer's instructions.

4.2.5. PCR amplification, cloning and sequencing

Bacterial 16S rRNA genes including were amplified using the universal primers 27F and 1492R (Lane, 1991). Briefly, the PCR was conducted in a total volume of 50 µl containing 2 µl of DNA, 0.5 µl of each primer (10 pmol), 1 µl of Ex-*Taq* polymerase (2.5U/ µl) (Takara BioInc., Korea), 4 µl of dNTP mix (2.5 mM each), and 5 µl of 10X buffer provided with the enzyme, under the following conditions: an initial denaturation step at 94°C for 5 min followed by 25 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1.5 min.

For the detection of DsrAB genes, genomic DNA from microcosm set were amplified using the DSR 1F/4R primer set (Wagner *et al.*, 1998). Briefly, the PCR was conducted in a total volume of 50 µl containing 2 µl of DNA, 0.5 µl of each primer (10 pmol), 1 µl of Ex-*Taq* polymerase (2.5U/ µl) (Takara Bio Inc., Korea), 4 µl of dNTP mix (2.5 mM each), and 5 µl of 10X buffer provided with the enzyme, under the following conditions: an initial denaturation step at 94°C for 3 min followed by 25 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 1 min. All primer information are shown in Table 4.2.

The purified PCR products were inserted into the vector using the TOPO TA cloning kit (Invitrogen, CA, USA). Fifty clones for 16S rRNA genes and twenty clones for DsrAB genes were randomly selected and sequenced

using M13F/M13R primers with ABI 3700 capillary sequencer (Applied Biosystems, CA, USA).

4.2.6. Quantitative real-time PCR

The extracted DNA from five samples (O1, O2, B1, B2 and B3) were used for SYBR green assays as a template. Quantitative PCR (qPCR) was performed to quantify the total numbers of gene copies using the Rotor-Gene Q Real-Time PCR system (Qiagen, Hilden, Germany). Briefly, the amplification was performed in a total volume of 20 µl reaction mixture containing 2 µl of DNA, 1 µl of primers (10 pmol each), and 2× SYBR Green Master Mix (Enzynomics, South Korea) under the following conditions: an initial denaturation at 95°C for 10 m, 40 cycles of 10 s at 95°C, 15 s at 60°C and 20 s at 72°C, and the melting curve with same profile. The SYBR Green assays were conducted with the primer and probe sets targeting 16S rRNA genes of universal bacteria and *Desulfosporosinus* sp. (Table 4.2). The standard curves were established using 10-fold serial dilutions of plasmid DNA containing 16S rRNA gene and DsrAB gene clones from untreated FRX and microcosm samples. The serial dilution was ranging from 10^0 to 10^6, and R square for standard curves was 0.98-0.99. Gene copy numbers were calculated per ml of FRX and microcosm samples as described (Ritalahti *et al.*, 2006).

4.2.7. 16S rRNA gene amplicon sequencing, community and phylogeny analysis

The V3-V4 region of 16S rRNA gene from five samples (O1, O2, B1, B2 and B3) were amplified with the primer pair (Zheng *et al.*, 2015) (Table 4.2), using the 250 bp paired-end, Illumina® MiSeq platform (Macrogen, Korea). Primer sequences and index information were trimmed, and low-quality sequences were filtered out from the dataset using Sickle and Spades (Bankevich *et al.*, 2012), respectively. Paired-end assembly was conducted with Pandaseq platform (Masella *et al.*, 2012), and the quality-filtered sequences were further processed following the MiSeq SOP using mothur (Kozich *et al.*, 2013). The partial 16S rRNA gene sequences originated from bacteria were clustered into operational taxonomic units (OTUs) with 97% similarity cutoff using mothur. Taxonomic classification was assigned using the Naïve Bayesian Classifier implemented in mothur against the EzTaxon-e database (http://eztaxon-e.ezbiocloud.net) (Kim *et al.*, 2012, Kozich *et al.*, 2013).

For the construction of phylogenetic tree, the previously identified partial 16S rRNA gene and DsrAB gene sequences were used with thirteen (for 16S rRNA gene) and twelve (for Dsr gene) clones from this study. All sequences were aligned using Muscle algorithm (v3.8.31) (Edgar, 2004). The aligned sequences were manually edited using Bioedit and they were used to infer the phylogeny tree based on Neighbor-joining algorithm in MEGA7. Bootstrap value was tested in 1,000 replications and the evolutionary distances

were computed using Jukes-cantor method. The *Firmicutes* isolate (AM183354) and archaea DsrAB gene were used as an outgroup of the trees for 16S rRNA gene and DsrAB gene, respectively.

4.3. Results

4.3.1. Evidence of sulfate reduction in microcosm

Visible turbidity and colorimetric change were achieved from only one condition among microcosm sets after 65 days of cultivation under anaerobic condition. While the concentration of sulfate was gradually reduced from 8.33 mM at Day-6 to 1.04 mM at Day-65, except for the first 6 days of the incubation (6.25 mM at Day-0), those of sulfide gradually increased from noneto 0.37 mM by Day-65 in microcosm amended with 1 mM lactate and 5 mM sulfate to FRX water at 10°C (Figure 4.3).

4.3.2. Shifts in bacterial community and identification of dominant populations during the anaerobic incubation

Total of 679,424 high-quality sequences, ranging from 124,079 for B1 to 163,502 bp for B3, was recovered from five samples. Sequences were clustered into 1,621 OTUs classified with 52 phyla. After 65 days of incubation, the relative abundances of bacteria in phylum/sub-genus level were compared among five samples (two from raw FRX lake samples and three from

microcosm) to evaluate the response of bacterial community structures (Figure 4.4). In phylum level, the raw FRX samples (O1 and O2) were dominated by *Gammaproteobacteria* (57% for both of O1 and O2), followed by *Firmicutes* (22% and 23%), *Actinobacteria* (3%), OD1 (3%), JS1 (2%) and *Chloroflexi* (2%). After 65 days of incubation, *Gammaproteobacteria* was still predominant, but slightly reduced ranging from 43% in B1 to 22% in B3. Also, *Bacteroidetes* rapidly increased from 1% (O1 and O2) to 30% (B2), and *Firmicutes* and *Spirochaetes* increased to 30% and 5%, respectively.

To better understand shifts in community structure, the relative abundances were analyzed at the sub-genus (OTU) level in fine scale (Table 4.3). In the raw FRX samples (O1 and O2), three of genus were dominant with more than 20% in abundance, which were *Thiomicrospira* (*Gammaproteobacteria*) (28.4%), *Psychrobacter* (*Gammaproteobacteria*) (27.7%) and *Paenisporosarcina* (*Firmicutes*) (21.9%). After enrichment (B1, B2 and B3), three genera *Desulfosporosinus* (27.1%), *Psychrobacter* (21.4%) and *Thiomicrospira* (12.9%) belonging to *Firmicutes* were predominant. Interestingly, the genus *Desulfosporosinus* belonged to *Firmicutes* was promptly soared from 0.1% (O1 and O2) to 28% (B1, B2 and B3). Also, four genera increased (0.01% for all of four in the initial samples) in enriched samples, GU061294_g (10.7%), FJ269057_g (6.2%), and AJ853611_g (*Bacteroidetes*) (11.0%), and *Sphaerochaeta* (*Spirochaetes*) (4.4%). These OTU sequences based major changes were compared with public available

sequences against Genbank database (Table 4.4). The sequence of OTU_18055 was well matched with genus *Desulfosporosinus* as well.

4.3.3. Quantification of bacteria and targeted population in FRX and microcosm samples

To evaluate the extent of the change of bacteria population between untreated and enriched samples, 16S rRNA gene copies overall bacteria and specifically, the genus *Desulfosporosinus* were quantified using qPCR assay. In the initial samples (O1 and O2), 16S rRNA gene copies of total bacteria were detected with concentration of 1.46×10^{9} and 1.33×10^{9}, respectively, and those of the genus *Desulfosporosinus* were detected with low abundance ranging from 1.20×10^{2} to 1.80×10^{2} (Figure 4.5). In the microcosm samples (B1, B2 and B3), 16S rRNA gene copies of total bacteria were reduced than the initial samples ranging from 2.33×10^{8} (B1) to 8.01×10^{7} (B3), after 2-month incubation. Interestingly, concentration of the genus *Desulfosporosinus* was notably increased from 1.37×10^{7} (B1) to 5.24×10^{6} (B3). The ratio between total bacteria and the genus *Desulfosporosinus* was shown zero in initial sample, but its proportion within bacterial cells was raised to 6% (B2) and 10% (B1) through enrichment of *Desulfosporosinus*.

4.3.4. Phylogeny diversity of 16S rRNA gene and DsrAB gene in *Desulfosporosinus*

Phylogenetic analysis based on the full-length 16S rRNA gene sequences and DsrAB gene indicated that these lineages are ramified from previously identified *Desulfosporosinus* (Figure 4.6). All clones of two genes retrieved from SRB microcosm of FRX were most closely affiliated with *Desulfosporosinus* sp. strain 63.6F (KX822015) sequence, but there was a distance among them by clustering different clades from *Desulfosporosinus* groups.

4.4. Discussion

This is the first microcosm study for SRB combined with high-throughput amplicon sequencing in FRX of McMurdo Dry Valleys, Antarctica. The diversity of SRB in FRX have been reported using traditional culture-dependent and fingerprint approaches (Smith *et al.*, 1993, Karr *et al.*, 2005). This study also performed microcosm test, which used the lake water as a medium and has provided new growth condition targeting not yet enriched SRB.

Enriched samples from FRX showed evidences of sulfate reduction according to concentration change of sulfate and sulfide (Pester *et al*. 2010). When SRB use lactate, formate or acetate as energy sources, they also consume sulfate as an electron acceptor and emit sulfide as a final product in metabolic

pathway of sulfate reduction. These results could indirectly indicate that SRB have been enriched and have active role in the microcosm.

Through high-throughput sequencing and quantification method, the incubation for 65-days corroborated that the genus *Desulfosporosinus* was the main microorganism of SRB in microcosm, which was consistent with preceding study (Spring & Rosenzweig, 2006, Pester *et al.*, 2010). The 16S rRNA gene copies of *Desulfosporosinus* belonging to the phylum *Firmicutes* were remarkably enriched in all three samples, and this result confirmed physiological activity of *Desulfosporosinus* in microcosm (Pester *et al*. 2010). It also indicated that medium conditions based on lake water in microcosm selectively supported growth effect to the genus *Desulfosporosinus.* The enriched *Desulfosporosinus* also showed their adaptation in FRX ecosystem in respect to their ecotype in phylogenetic tree.

The genus *Desulfosporosinus* was defined as a member of the 'rare biosphere' consisting of abundance below 0.1% in the total communities in natural abundance (Sogin *et al.*, 2006, Fuhrman, 2009). Despite of their low abundance, *Desulfosporosinus* population has been highly considered as important because of their possibility to drive extensive part of sulfate reduction (Pester *et al.*, 2010). They effectively reduced sulfate despite of their low abundance, so called high cell-specific reduction (Pester *et al*., 2010). *Desulfosporosinus* was also reported as versatile organism because they could be a fermenter of lactate and pyruvate under sulfate limitations (Spring and

Rosenzweig, 2006) through reductive acetogenesis (Rabus *et al*, 2006) or dissimilatory iron (III) reduction (Ramamoorthy, 2006). Moreover, they are well sustained under droughts and oxygenation condition by forming endospores, which indicate that they may well adapt to low sulfate of extreme environments (Spring and Rosenzweig, 2006). At the same time, these characteristics provide clues that how they were remarkably enriched in specific condition from extremely low abundance in the samples.

Members of the genus *Desulfosporosinus* were grown under strict anaerobe condition, and found in a variety of habitats such as low-sulfate freshwater, rock drainage, pristine aquifers, municipal drinking water, and permafrost (Vatsurina *et al.*, 2008). The hallmark feature of members of the genus *Desulfosporosinus* could be autotrophic growth with H_2, and fermentate the lactate under sulfate limitation (Spring and Rosenzweig, 2006). Rabus *et al*. reported that these autotrophic sulfate reducers display faster doubling times than heterotrophic species (Rabus *et al.*, 2006). In addition, they are able to use a wide range of electron donors (such as formate, pyruvate, acetate with H_2, and butyrate), and diverse sulfide compounds as electron acceptors (sulfur, sulfite, thiosulfate, sulfate, and tetrachlorethene) (Spring and Rosenzweig, 2006). These findings support that how the genus *Desulfosporosinus* were able to be autonomously enriched during the incubation period in given condition of the this study.

Otherwise, several populations based on OTUs were also incubated in

microcosm. GU061294_g (OTU_15227) belonging to the family *Flavobacteriaceae*, which was grown from 0.1% to 10% during incubation, was closely related to the genus *Lutibacter* with 96% similarity. Benthic *Flavobacteriaceae* family dominated by the genus *Lutibacter* was detected in impermeable sediments (Probandt *et al*., 2017). They are aerobic and facultative anaerobic organoheterotrophs and could respire carbohydrates (Choi *et al.*, 2013).

FJ269057_g (OTU_22670) was increased in abundance ranging from 3.2% to 8.4% after incubation, which showed highly matching with genus *Solitalea* with 97% similarity. This genus is known to reduce sulfates to hydrogen sulfide (Krieg, 2010). *Solitalea*-related population is related to consumption of the organic substrates for sulfate reduction in the later cycles (Jung *et al.*, 2016).

AJ853611_g (OTU_29067) also grew with its abundance ranging from 4.85 to 20% after incubation. The genus *Lentimicrobium* was the closest taxa with 97% similarity. This genus was isolated from methoanogenic sludge treating starch-based organic wastewater under anaerobic conditions (Sun *et al.*, 2016). It grew chemoorganotrophically on a narrow range of carbohydrates (Sun *et al*., 2016).

The genus *Sphaerochaeta* (OTU_00255) was increased in abundance, up to approximately 4% after incubation, which had form of free-living sulfate reducer. It belonged to fermentation-capable *Spirochaetes* phylum isolated

from anaerobic environments (Ritalahti *et al.*, 2012). The genus *Sphaerochaeta* ubiquitously existed from hypolimnion of Antarctic Ace lake (Franzmann & Dobson, 1992), dechlorinating enrichment cultures (Li *et al.*, 2013), freshwater sediments (Ritalahti *et al.*, 2012), and hypersaline Antarctic lake (Murray *et al*., 2012).

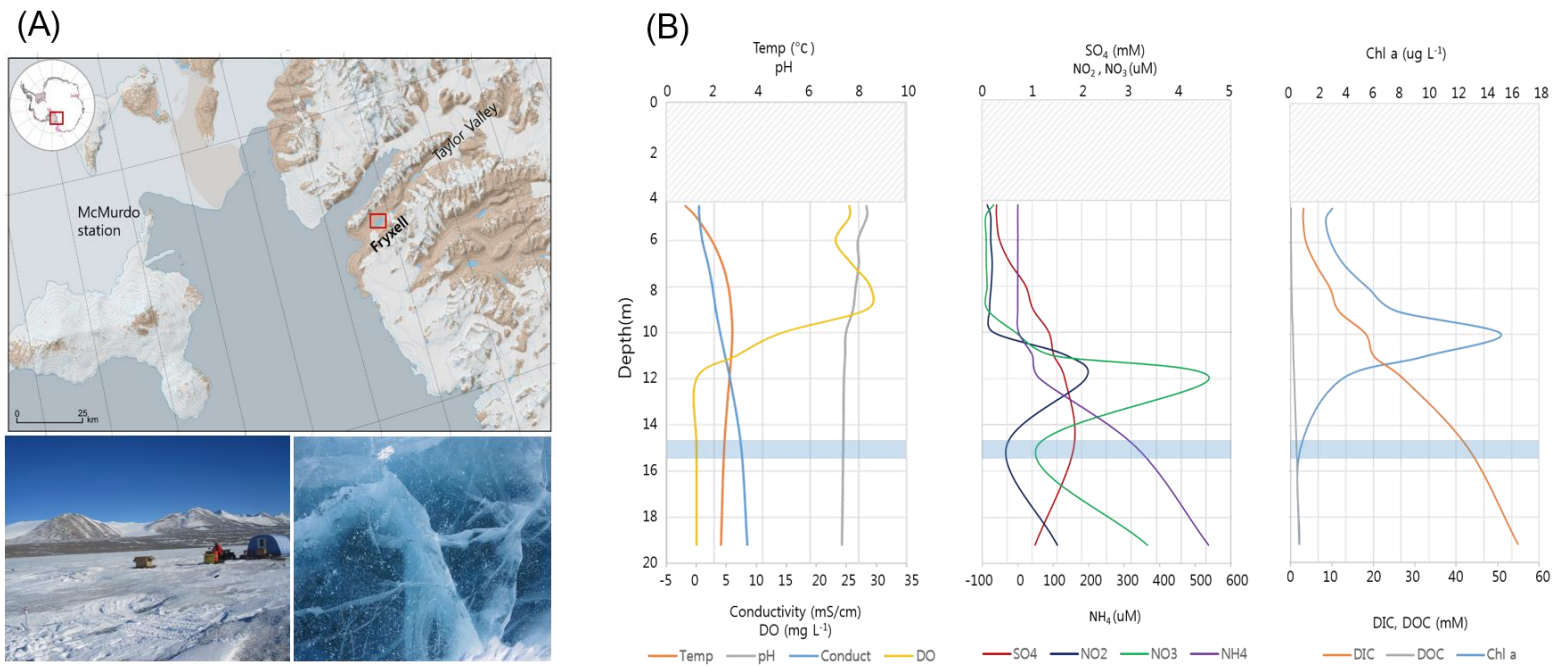

Figure 4.1. Site map for Lake Fryxell in Taylor Valley, Antarctica. (A) site description (B) and biogeochemical depth profiling.

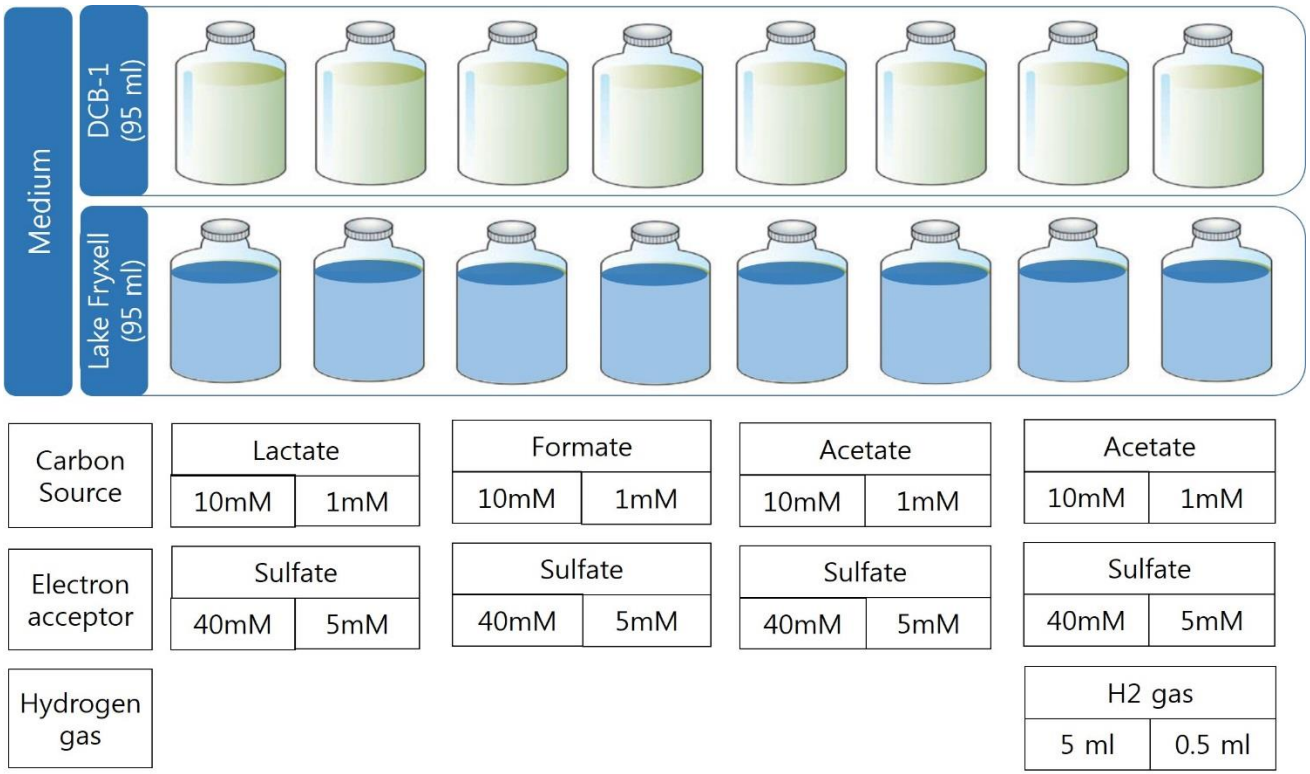

Figure 4.2. Microcosm setups of sulfate reduction and its transfers with Lake Fryxell sample. Five mililiter of Lake Fryxell water were inoculated into all serum bottles. Each medium was incubated at 4°C and 10°C under the dark condition.

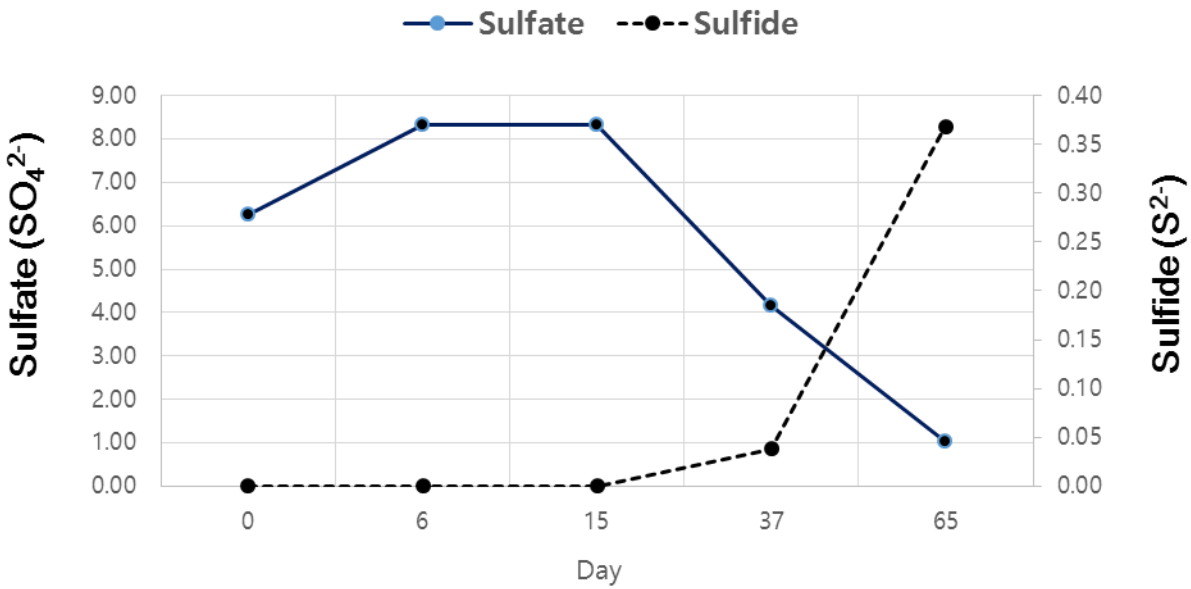

Figure 4.3 Monitoring of sulfate and sulfide concentration during incubation in microcosm.

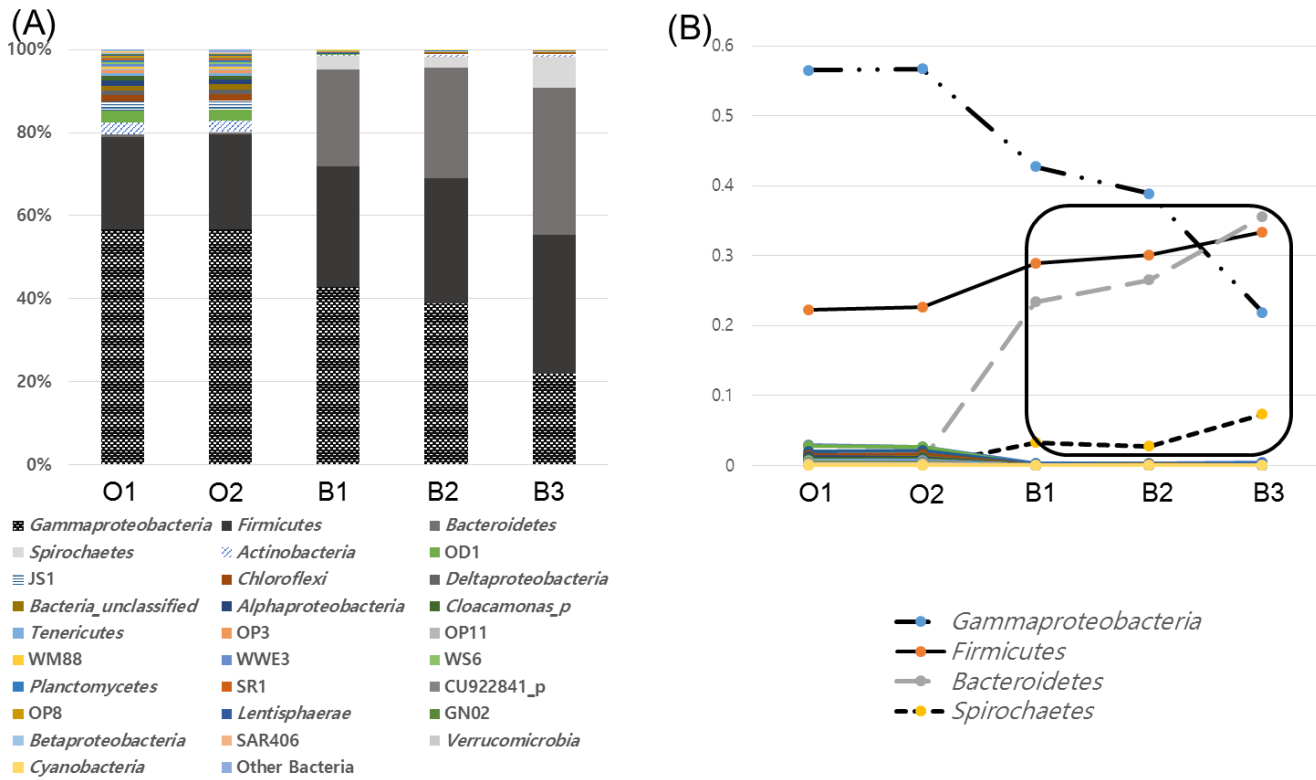

Figure 4.4 Changes of represented bacterial community in relative abundance detected in Lake Fryxell and microcosm sample of Lake Fryxell. (A) bacterial community structure in phylum level and (B) top four phyla in major change.

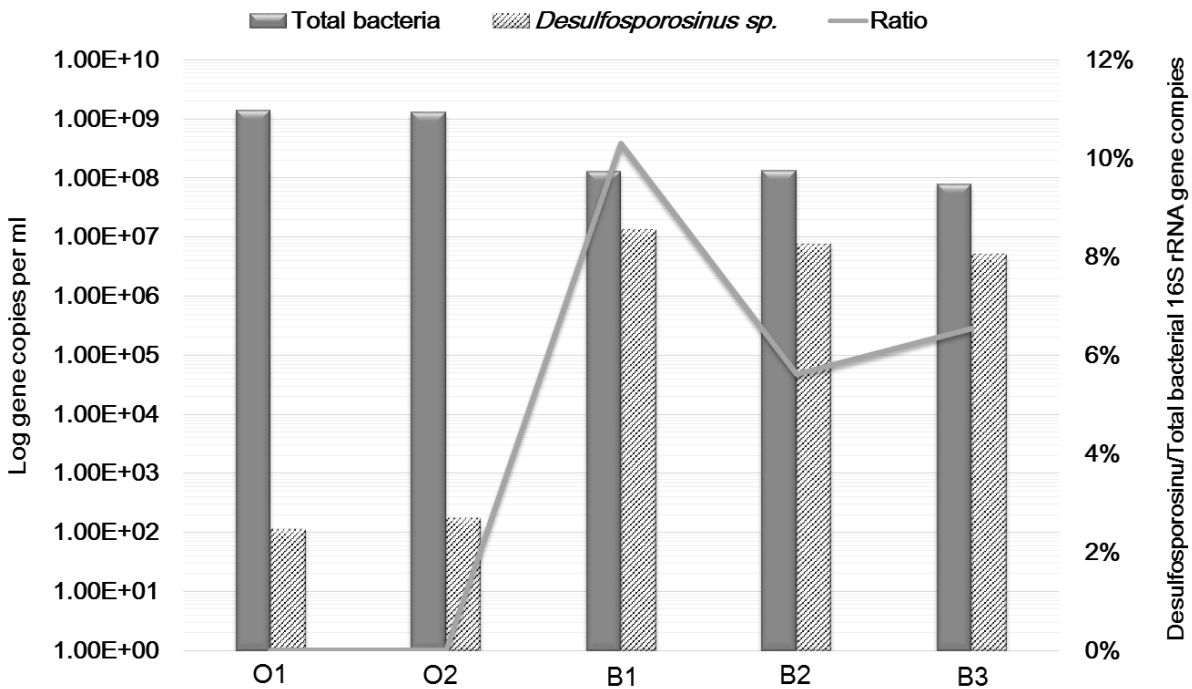

Figure 4.5 16S rRNA gene-based qPCR results based on SYBR green assays for total bacteria and *Desulfosporosinus* in Lake Fryxell (O1 and O2) and their sulfate reduction microcosms (B1, B2, and B3). The numbers of gene copies of total bacteria and *Desulfosporosinus* in left axis, and the ratio of two measurements within sample in right axis.

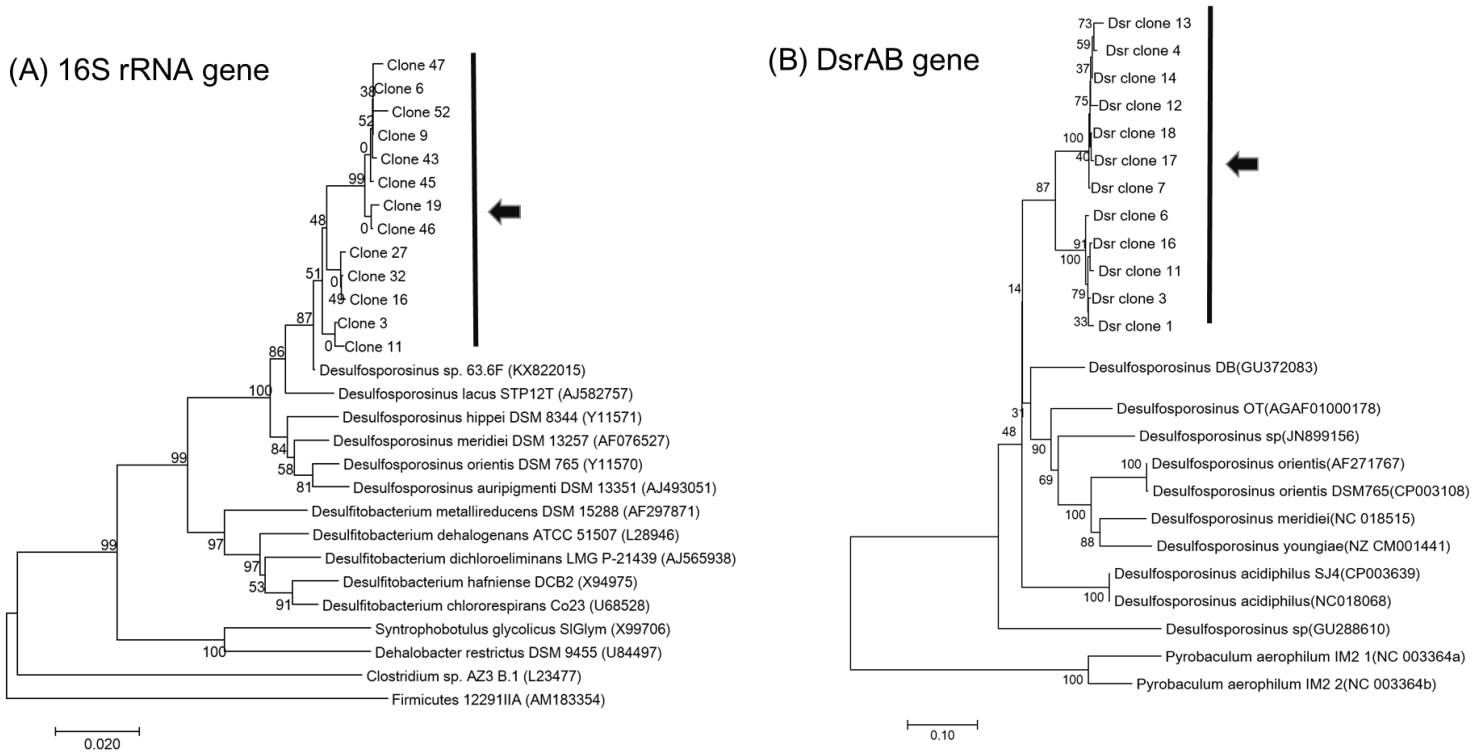

Figure 4.6 Phylogenetic tree of (A) 16S rRNA gene and (B) DsrAB gene obtained using *Desulfosporosinus* group-specific primers from microcosm sample of Lake Fryxell.

Table 4.1 Physicochemical and biological characteristics at 15 m of FRX.

Lake	Month	Depth (m)	Physical				Biological		Chemical			
			Cond. (mS cm^{-1})	Temp	UwPAR (umol phot/ m2/s)	Amb PAR (umol phot /m2/s)	BacPro (nM TDR/ day)	Chl-a (ug chl-a/ L)	SO4 (mM)	DIC (mM)	DOC (mM)	pH
FRX	Nov	15	7.4336	2.447	0.02	1050	0.0004	0.85	1.83	40.6	1.26	7.48
			Ions (mM)									
			Li	Na	K	Mg	Ca	F	Cl	Br	Si	
			0.0182	87.1	4.02	9.85	3.04	0.26	76.8	0.1	0.562	

Table 4.2 Oligonucletide primers used for PCR and real-time PCR amplification of 16S rRNA genes for universal bacteria and *Desulfosporosinus*, and DsrAB gene.

	Primer name	5'-sequence-3'	Target	Reference
PCR	27F	AGAGTTTGATCMTGGCTCAG	16S rRNA gene	Lane (1991)
	1492R	GWATTACCGCGGCKGCTG	16S rRNA gene	Lane (1991)
	DSR1F	ACSCACTGGAAGCACG	DsrAB gene	Wagner (1998)
	DSR4R	GTGTAGCAGTTACCGCA	DsrAB gene	Wagner (1998)
Real-time PCR	Bac1055YFa	ATGGYTGTCGTCAGCT	16S rRNA gene	Ritalahti et al (2006)
	Bac1392R	ACGGGCGGTGTGTAC	16S rRNA gene	Ritalahti et al (2006)
	Desulfo16S_246F	CCTTGGAAACGAGGGCTAATAC	*Desulfosporosinus*	In this study
	Desulfo16S_347R	CGCGGGTCCATCCATAATC	*Desulfosporosinus*	In this study
Miseq	341F	CCTACGGGNGGCWGCAG	16S rRNA gene	Zheng et al (2015)
	805R	GACTACHVGGGTATCTAATCC	16S rRNA gene	Zheng et al (2015)

Table 4.3 Identification of major changed in bacterial populations in OTU level during the incubation.

No. of OTU	Phylum	Class	Order	Family	Genus	O1	O2	B1	B2	B3
OTU_01253	*Proteobacteria*	*Gammaproteobacteria*	*Thiomicrospira_o*	*Thiomicrospira_f*	*Thiomicrospira*	28.2%	28.7%	15.9%	17.5%	5.2%
OTU_03143	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Psychrobacter*	27.9%	27.5%	26.6%	21.1%	16.6%
OTU_12962	*Firmicutes*	*Bacilli*	*Bacillales*	*Planococcaceae*	*Paenisporosarcina*	21.8%	22.0%	0.0%	0.0%	0.0%
OTU_18055	*Firmicutes*	*Clostridia*	*Clostridiales*	*Desulfitobacterium_f*	*Desulfosporosinus*	0.0%	0.1%	28.8%	28.8%	23.7%
OTU_29067	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	FJ437992_f	AJ853611_g	0.0%	0.1%	4.8%	8.3%	20.0%
OTU_22670	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	FJ437992_f	FJ269057_g	0.0%	0.0%	7.1%	8.4%	3.2%
OTU_15227	*Bacteroidetes*	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*	GU061294_g	0.1%	0.1%	11.2%	9.5%	11.6%
OTU_00255	*Spirochaetes*	*Spirochaetes_c*	*Spirochaetales*	*Spirochaetaceae*	*Sphaerochaeta*	0.0%	0.0%	3.3%	2.7%	7.3%

Table 4.4 BLAST results of OTU sequences in major enriched bacteria against Genbank database

No. of OTU	Accession	Taxonomy of closest match					Query cover	Similarity
		Phylum	Class	Order	Family	Genus		
OTU_18055	KX822015.1	*Firmicutes*	*Clostridia*	*Clostridiales*	*Peptococcaceae*	*Desulfosporosinus*	100%	99%
OTU_29067	LC049960.1	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Lentimicrobiaceae*	*Lentimicrobium*	100%	97%
OTU_22670	LC049960.1	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Lentimicrobiaceae*	*Lentimicrobium*	100%	97%
OTU_22670	KF528160.1	*Bacteroidetes*	*Sphingobacteriia*	*Sphingobacteriales*	*Sphingobacteriaceae*	*Solitalea*	100%	97%
OTU_15227	NR_116738.1	*Bacteroidetes*	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Lutibacter*	100%	96%
OTU_00255	HG531807.1	*Spirochaetes*	*Spirochaetes_c*	*Spirochaetales*	*Spirochaetaceae*	*Sphaerochaeta*	100%	96%

CHAPTER 5.

General Conclusions

Antarctica has been considered as extreme conditions in respect to microbes because of pristine and harsh environments. The ice-covered lakes in Dry Valleys, inter alia, which are known as the driest, coldest, and truncated environments in the Antarctic continents, provide habitats to diverse microbes. Many scientists have been interested in the Antarctic area because it would provide valuable clues for climate change and life history on the earth. In recent years, with the development of technologies tools and analysis methods, biogeochemical characteristics and microbial diversity have been reported. However, bacterial spatial distribution and their ecological functions are still poorly understood owing to small sample size and confined access to the Antarctic environment. This study investigated how bacterial communities differ within ice-covered lakes in McMurdo Dry Valleys, Antarctica, and discussed their bacterial communities and their functions, especially sulfate reducing bacteria in Lake Fryxell.

Firstly, bacterial community structures were investigated and were compared among the ice-covered lakes, which exhibited different biogeochemical characteristics. The hypothesis of uncharted branches and niche bacterial lineages in unique environmental conditions were tested within and among the lakes. The upper layers of lakes have mostly shown similar bacterial communities. In contrast, the deep waters beneath the chemoclines supported highly distinct communities both within each lake and among lakes, most likely due to spatial isolation and the unique geochemistry between lakes,

and within the strata of each individual lake. More diverse taxa, including candidate divisions, were detected in the bottom of Lake Fryxell, which was reported to have active sulfate reduction to be occurring. Community structures were strongly related with biogeochemical factors by depth, lake, and bacterial size fractions.

Secondly, key players in sulfate reduction and their potential ecological functions in the bottom depth of Lake Fryxell were further examined based on culture-independent approach, for example, metagenome shotgun sequencing. Draft genomes were retrieved from metagenome data, including candidate phylum and sulfate reducer. *Deltaproteobacteria* genomes were suggested as main players of sulfate reducers according to their metabolic abilities. The candidate division WM88, which dominantly exists in the bottom of the lake, was suspected to indirectly contribute to sulfur cycle. The ecological functions of uncultured bacteria were observed from the draft genomes in environment samples without cultivation.

Lastly, active players of sulfate reduction in the bottom depth of Lake Fryxell were examined through culture-dependent approach of microcosm cultivation. Physicochemical similarity between medium and real environment reproduced suitable growth environment for bacteria, which reside in the environment. This hypothesis was tested using lake water as medium in microcosm set up. The genus *Desulfosporosinus* was selectively enriched in high ratio in specific conditions of temperature and concentration of carbon

source, which is well known as sulfate-reducing bacteria. Interestingly, the genus *Desulfosporosinus* have been considered as an important player of sulfate reduction due to their effective capacities, and this genus were reported as a member of the ‘rare biosphere’.

Over all, this study generated several novel results that may provide further insights into the ecology and evolution of bacteria inhabiting in the ice-covered lakes of Antarctica. The presence of these niche-specific lineages provides evidence of strong specialization of bacteria, which have uniquely adapted to isolated lake ecosystems over a long period of time. More integrated approaches, such as single-cell genomic and other omic technologies, are needed to further examine the links between bacterial taxa and metabolic functions in these unique ecosystems.

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국문초록 (Abstract in Korean)

남극은 극한 환경조건들로 인해 미생물이 살기 어려운 환경일 뿐 아니라, 현재까지 원시적 환경을 유일하게 간직하고 있는 곳으로 알려져 있다. 그 중에서도, 남극의 영구적으로 얼음이 덮힌 호수들은 화학적 및 생물학적 요소에 의해 뚜렷한 성층현상을 보이고 있다. 이러한 독특한 환경으로 인해 많은 연구자들이 오랜기간 관심을 가져왔음에도 불구하고, 물리화학적 자료에 비해 미생물의 경우, 다양성에 대한 기초자료조차 부족한 실정이다.

본 연구에서는 얼음이 덮힌 호수들 중 대표적인 다섯 개 호수를 선정하여 16S rRNA gene을 기반으로 파이로시퀀싱이라는 차세대 시퀀싱 기술을 이용하여 박테리아 다양성 분석 및 비교하고자 하였다. 그 결과, *Bacteroidetes, Actinobacteria 및 Proteobacteria* 같은 우점종을 제외하고, 각 호수들마다 서로 다른 박테리아 군집 구조를 나타내었고, 특히 심층부로 갈수록 군집구조의 차이가 확인히 나타났다. 이는 생지화학적 요소가 다른 각 호수들의 환경적 특징을 반영해주는 결과였다. 또한, 특정 박테리아 lineage들은 호수 내에서도 특정 수 층에 국한되어 검출되었다. 예를 들면, candidate phylum WM88은 프릭셀 호수의 15 m 깊이에서 발견되었다. *Chlorobi*의 특정

genus 경우 미얼스 호수의 18 m 깊이에서 발견되었고, *Firmicutes*와 *Gammaproteobacteria*의 서로 다른 genus가 이스트바니와 웨스트바니 호수의 30m 깊이에서 각각 서로 다르게 발견되었다. 가장 독특하면서도 다양한 박테리아를 보여 준 호수는 프릭셀 호수의 15 m 깊이로, 다른 호수들과 달리, candidate phylum의 높은 우점도 뿐 아니라 다양한 minor candidate phyla들이 존재하고 있었다.

프릭셀 호수의 심층은 기존 연구들을 통해 황산염 환원이 많이 일어나고 있는 호수로 알려져 왔다. 하지만, 프릭셀 호수의 황산염 환원 박테리아에 대한 다양성 정보는 여전히 부족 한 실정으로, 검출 된 군집정보와 환경요소 간의 연관성 연구가 필요하다. 현재까지 전체 박테리아 중 약 1%만이 배양 가능한 한계점이 있기 때문에, 메타지놈 분석방법을 적용하여 환경에 존재하는 박테리아의 전체 군집 및 기능을 알 수 있다. 따라서, 프릭셀 호수 15 m의 황산염환원 박테리아를 대상으로 비배양법을 적용하여 지놈 정보를 얻고, 이들의 생태학적 역할을 잠재적으로나마 알고자 하였다. 그 결과, 황산염 환원 관련 3개의 지놈을 추출하였고, 그 중, 개 지놈은 *Deltaproteobacteria,* 1개 지놈은 candidate division WM88에 속하는 것을 확인하였다. 황산염 환원 과정에 중요한 역할을 하는 필수 효소들의 존재유무를 통해, *Deltaproteobacteria*들이 주요 황산염 환원자들로 판

단되었고, WM88의 경우 필수 효소들이 발견 되지 않았지만, 황산염 전환, 수소 및 전자운송 관련 효소들을 보유한 것으로 보아 황산염 환원에 간접적인 역할을 할 것으로 판단되었다. 그 외의 대사 과정을 살펴보았을 때, 남극 호수라는 빈영양 호수에서 발견 된 만큼, 모두 다양한 에너지 보존 및 사용에 대한 능력을 보유 하고 있는 것으로 판단되었다.

비배양방법으로 전체적인 군집 및 기능의 정보를 알 수 있었으나 실질적인 활성 가능성을 확인하기 어렵기 때문에, 프릭셀 호수 15 m 샘플에서 황산염 환원 박테리아를 대상으로, 비배양법과 동시에 배양방법을 적용하였다. 호수의 실제 수 시료를 배지로 사용하는 마이크로코즘 방법을 이용한 결과, *Desulfosporosinus* 속이 유일하게 매우 뚜렷하게 증식 된 것을 확인하였다. 이 속은 황산염 환원자로 알려져 있을 뿐 아니라, 실제 환경에서 매우 적은 양으로 존재하는 ‘rare biosphere’에 속하는 것으로 알려져 있다. 기존 연구들을 통해 이들이 적은 비율로 환경에 존재할지라도 황산염 환원 능력이 뛰어난 것이 알려지면서 황산염 환원자로써의 중요성이 평가되어지고 있다.

남극 호수에서의 박테리아 군집 구조와 생태학적 역할에 대한 연구 결과는 대용량 시퀀싱, 오믹스 및 배양이라는 다양한 방법

을 접목하여 이끌어 낸 첫 사례이다. 도출해 낸 결과들은 남극과 같이 극한이면서도 독특한 생지화학적 요소를 지닌 환경에서의 박테리아의 적응, 환경요소와의 관련성 및 진화에 대한 중요 자료로써 이용 가능할 것으로 보인다.

주요어: 미생물 다양성, 개체 특수화, 황산염 환원 박테리아, 파이로시퀀싱, 메타지놈, 마이크로코즘, 프릭셀, 남극호수

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